Vol. 98, No. 3 June, 1950

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

METABOLIC ACTIVITIES AND CLEAVAGE OF EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA
A REVIEW, 1932–1949 [1]

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A. Introduction

The literature on the chemical and physical properties of eggs of *Arbacia punctulata* was summarized by Harvey (1932). During the ensuing period, the use of eggs of Arbacia and other sea urchins as representative cells for the study of the fundamental properties of animal tissue has greatly increased.

Two problems, closely related to each other, have been extensively studied: the analysis of the chemical events associated with fertilization; and the elucidation of the chemical processes by which energy liberated by oxidation of foodstuffs is made available for mitotic processes and cleavage.

The first of these two problems is the subject of excellent reviews by Tyler (1948) and Runnström (1949) and will therefore be dealt with here only incidentally.

The second problem is constantly gaining in interest because of its fundamental relationship to normal and pathological growth processes. As the result of investigations by both American and European workers there is probably no cell or tissue, with the possible exception of skeletal muscle, for which information relating enzymatic processes to function is as complete as it is for the sea urchin egg.

The principal objective of this paper is to bring together what is known about the relation of metabolic enzymes and metabolic processes to cleavage in the Arbacia egg. In addition, because of their potential bearing on this objective, the effects of chemical agents upon metabolic activity and cleavage are recorded; those agents which have known effects upon enzymes are stressed, but other agents are included. Chief attention will be given to the years 1932–1949 inclusive, publications prior to 1932 having been covered in the aforementioned review by Harvey (1932). Observations upon other marine eggs will be introduced where a comparison with the results on Arbacia is desirable.

Every effort has been made to leave no significant aspect of the relation of metabolic processes to cleavage unmentioned, but the bibliography does not include every publication in this field, as a given set of observations has in many instances been published in more than one place.

B. Oxygen Consumption

1. Units. Oxygen uptake of marine eggs has usually been expressed as c. mm. per 10^6 eggs or as c. mm. per hour per 10 c. mm. eggs. To permit expression of results in various units, the following data are useful.

Egg dimensions, average diameter, 74 μ; surface, 17,200 μ^2; volume, 212,000 μ^3 (Harvey, 1932). By diffractometer method, the mean volume is 228,000 μ^3 (Korr, 1937).

Density, 1.09 (Harvey, 1932).

Dry weight, 24.2 per cent of wet weight (Ballentine, 1940a); 23.9 per cent (Hutchens et al., 1942).

Nitrogen content, 0.107 mg. nitrogen per mg. dry weight, 5.86 mg. nitrogen per 10^6 cells (Ballentine, 1940a); 0.101 mg. nitrogen per mg. dry weight, 5.93 mg. nitrogen per 10^6 cells (Hutchens et al., 1942). Egg protein is assumed to be 6.25 times the egg nitrogen content.

Cells per mg. dry weight, 17,600.

Cells per 10 c. mm., 46,500.

Cells per wet gram, 4,300,000.

Weight of 10 c. mm. eggs, 10.9 mg. wet, 2.63 mg. dry.

2. Determination of Egg Volume. Measurements of egg volume have been commonly made by the hematocrit method. The hematocrit values obtained depend upon the period and intensity of centrifugation, and upon whether the volume which is measured is the total volume (eggs plus sedimentable jelly) or egg volume only. The most frequently used hematocrit value is that for the eggs only, as determined by centrifuging 5 minutes or more at 2000 × gravity or above, i.e. to an essentially constant volume. The egg volume as thus determined by hematocrit is about 8 per cent larger than calculated from hemocytometer measurements (Shapiro, 1935; Clowes and Krahl, 1936). The volume of a given number of eggs is 8 per cent smaller after fertilization than before (Glaser, 1924).

When results are expressed in terms of egg volume, it is helpful to state whether volume is determined on fertilized or unfertilized eggs.

3. Oxygen Consumption of Whole Eggs. Measurements on oxygen consumption of whole eggs are recorded in Table I.

The rate of oxygen consumption is strongly dependent upon the degree of trauma to which the Arbacia eggs are subjected prior to or during the measurement of oxygen consumption. Shaking rates above 48 per minute at an amplitude of 7.5 cm. (Whitaker, 1933a), light centrifuging during washing of eggs (Velick, 1941) or any other treatment which tends to break down the egg surface layers produces an increase in rate of oxygen consumption. Tyler, Ricci, and Horowitz (1938) found that the rise in oxygen consumption of unfertilized eggs, which begins some five hours after the eggs are shed, does not occur when the bacterial population in the sea water around the eggs is not allowed to increase.

For these reasons the absolute value of the oxygen consumption of *Arbacia punctulata* eggs cannot be specified except in relation to the conditions used for measurement. It follows that measurements designed to show effects of chemical agents and other treatments should be made under very carefully controlled and precisely described basal conditions. When the experimental conditions are kept constant, the values are reproducible from season to season.

Increase in oxygen consumption upon fertilization. The change in oxygen consumption of *Arbacia punctulata* eggs upon fertilization has been repeatedly studied since Warburg (1908) first showed oxygen consumption of the *Arbacia pustulosa* egg to be increased upon fertilization (Loeb and Wasteneys, 1911; Tang and Gerard, 1932; Whitaker, 1933a; Rubenstein and Gerard, 1934). The last named authors found that the factor by which oxygen consumption increases upon fertilization is a variable, its magnitude depending upon temperature. At 11° C., the fer-

TABLE I

Oxygen consumption of unfertilized and of fertilized eggs of Arbacia punctulata in natural sea water. The measurements are tabulated in chronological order. Where conversion factors were necessary, those given earlier in this paper were applied to the original measurements. A discussion of the validity of the numerical values is given in the text.

Observer	T° C.	Fert. (F) or unfert. (U)	Period of measurement, time after fertilization	Oxygen consumption
			hours	c. mm. per hr. per 10 c. mm. eggs
Tang (1931)	24.7	U	—	1.6 [1,2]
		F	0.25–1	7.9
Tang and Gerard (1932)	25	F	0.25–1	5.5 [1]
Whitaker (1933a, 1935)	21	U	—	0.4–0.5
		F	0.38–0.63	2.0
Gerard and Rubenstein (1934)	21	U [3]	—	0.9
Shapiro (1935a)	25.9	U	—	1.5
		F	—	3.9
Clowes and Krahl (1936)	20	U	—	0.6–0.7
	12	F	0.42–3	1.7
	20	F	0.42–3	3.1
	27	F	0.42–3	5.5
Korr (1937) [4]	15	U	—	0.2
	20		—	0.5
	25		—	1.0
	12	F	—	1.7
	15		—	2.1
	20		—	3.0
	25		—	4.7
Tyler, Ricci, and Horowitz (1938)	20	U	—	0.7
Krahl and Clowes (1938a)	20	F	0.42–3	3.1
Hutchens, Keltch, Krahl, and Clowes (1942)	20	F	1–4	2.4
			4–7	4.4
			7–11	4.9
			11–16	5.9
			16–26	6.8
Keltch and Clowes (1947)	20	F [5]	—	4.8

[1] The eggs were centrifuged lightly during washing, which tends to raise the oxygen consumption.

[2] Values are also given for various partial pressures of oxygen.

[3] Data are also given for change in oxygen consumption upon fertilization at temperatures from 11–29.9° C.

[4] The effects of pyocyanine, cyanide, and combinations of the two were also measured over the temperature range 11–26° C.

[5] Artificial parthenogenesis by hypertonic NaCl; 98 per cent membrane formation, 18–45 per cent cleavage.

tilized rate is about ten times the unfertilized; at 29.9° C., it is only twice that of the unfertilized.

At 20° C., the increase in oxygen consumption of Arbacia eggs is as large after artificial parthenogenesis (hypertonic NaCl) as after sperm fertilization (Keltch and Clowes, 1947). Mechanisms by which the increase in oxygen consumption upon fertilization may be brought about have been discussed by Whitaker (1933b), Krahl, Keltch, Neubeck and Clowes (1941), Goldinger and Barron (1946), Runnström (1949), Korr (1937), Ballentine (1940), and others. Whitaker (1933b) has summarized the measurements upon oxygen consumption of eggs of other marine forms: *Asterias forbesii, Cumingia tellinoides, Chaetopterus, Fucus vesiculosus, Nereis limbata.*

Oxygen consumption of the Arbacia egg increases gradually after fertilization until the egg is hatched, and then more markedly (Hutchens *et al.*, 1942). Similar studies on fertilized eggs of European sea urchins have been made by Gray (1927), Lindahl and Holter (1941), and Borei (1948). Zeuthen (1947) reported that, during the 5th to 8th cleavages of Psammechinus, the oxygen consumption is higher in the prophase, and lower in the later phases of a given mitotic cycle.

4. Oxygen Consumption of Egg Fragments. By high speed centrifuging in solutions of suitable specific gravity, unfertilized Arbacia eggs may be broken into a light half, containing the nucleus, the mitochondria, and some yolk; and a heavy half, containing the pigment granules and the major portion of the yolk. It has been found that the oxidative activity of the heavy half is somewhat greater than that of the light half, whether measured by oxygen consumption (Shapiro, 1935) or as reducing activity toward ferricyanide (Ballentine, 1940c). The combined oxygen uptake of the two halves of the unfertilized egg is 29 per cent greater than that of the whole egg, when proper allowance was made for volumes of the fragments; the sum of the respiratory rates of the two kinds of fertilized halves is 17 per cent less than that of the whole fertilized egg. Addition of p-phenylenediamine to the light halves produces a relatively smaller increase in oxygen consumption than addition of the same agent to the heavy halves (Boell *et al.*, 1940).

5. Respiratory Quotient. The respiratory quotient of unfertilized Arbacia eggs has apparently not been measured. That of the fertilized eggs appears to vary with the batch of eggs used; the rate of glycogen disappearance is low when the respiratory quotient is low. Observed R.Q. values are, for various periods after fertilization (Hutchens, Keltch, Krahl, Clowes, 1942): 1–4 hours, 0.85 (0.76–0.94); 1–7 hours, 0.88 (0.75–1.00); 1–11 hours, 0.90 (0.75–1.04); 1–16 hours, 0.88 (0.79–0.97); 1–25 hours, 0.86 (0.84–0.98). The respiratory quotient of the *Arbacia pustulosa* egg was found to be: unfertilized, 1.06; fertilized, 0.95 (Ashbel, 1930).

6. Oxygen Consumption of Sperm. See Barron, Seegmiller, Mendes, and Narahara (1948); Benedict and Barron (1946).

C. Rate of Cleavage

The times from fertilization to 50 per cent cleavage at 20° C. were observed by Fry (1936) to be: first cleavage, 69 minutes; second cleavage, 107; third cleav-

age, 145. For times to first cleavage at other temperatures, and times to other stages of development, see Harvey (1932).

Effects of various physical or chemical agents upon cleavage rate have been measured by two methods. In one, employed first by Smith and Clowes (1924), and used by Clowes, Krahl, and coworkers for their measurements, the control and experimental aliquots of eggs are allowed to develop until the control eggs have undergone several divisions; the eggs are then fixed in 0.1 per cent formaldehyde, and the percentage of the total in each state of division is determined by counting. The extent of division is expressed numerically according to the number of divisions undergone: no division, 0; 2 cell, 1; 4 cell, 2; 8 cell, 3; 16 cell, 4. For example, a population 25 per cent 4 cells, 70 per cent 8 cells, and 5 per cent undivided would have undergone, on the average, $0.25 \times 2 + 0.70 \times 3 + 0.05 \times 0 = 2.6$ divisions per egg. Cleavage in presence of various agents can be expressed as a fraction of control rate by dividing the divisions per egg in the experimental sample by the divisions per egg in the control.

In the second method of determining cleavage rate, the time to reach 50 per cent first cleavage is determined by serial fixation and counting of samples. The effect of experimental conditions can then be expressed as minutes of cleavage delay (or acceleration), as ratio of time required for the experimental sample to that required for control, or as per cent delay or acceleration relative to the control rate.

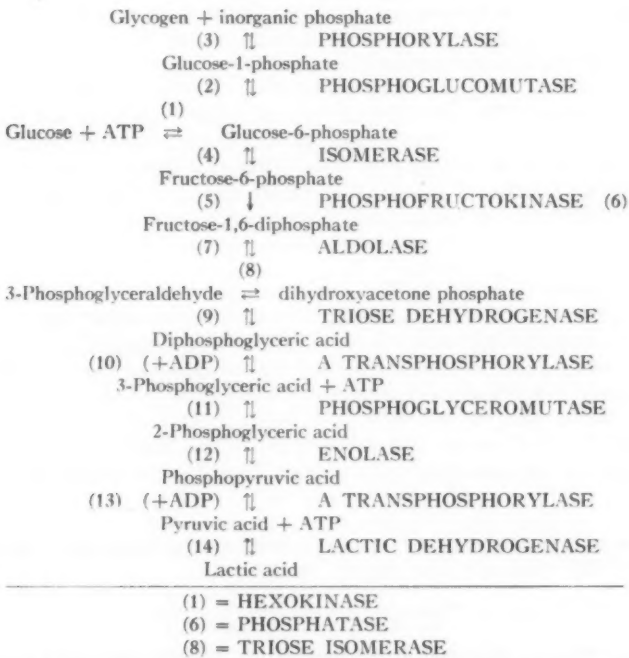

FIGURE 1. Steps in anaerobic breakdown of glucose or glycogen in animal tissues.

D. Metabolic Catalysts, Metabolic Intermediates; Phosphorus, Nucleic Acid and Calcium Metabolism; Echinochrome

1. Metabolic Catalysts. The search for metabolic catalysts in the sea urchin egg has been guided by previous observations on yeast and skeletal muscle. According to current concepts, carbohydrate is apparently broken down by muscle in two phases; in the first (anaerobic) phase, glucose or glycogen is broken down to pyruvate or to a 2 carbon fragment (Fig. 1). In the second (aerobic) phase, the carbons of the pyruvate or 2 carbon fragment are converted to carbon dioxide via the reactions of the tricarboxylic acid cycle (Fig. 2). The hydrogens removed during the aerobic phase are transferred, over diphosphopyridine nucleotide or triphosphopyridine nucleotide, flavin-adenine dinucleotide, and the cytochromes to react finally with oxygen and form water (Fig. 3); the hydrogens removed during the anaerobic cycle are used to reduce pyruvate to lactate. Amino acids employed as foodstuffs are first deaminated and then oxidized over the tricarboxylic acid cycle. Fatty acids to be oxidized are broken down to 2 carbon fragments which then also enter the tricarboxylic acid cycle (see Sumner and Somers, 1947; Krebs, 1943; Ball, 1944; and Lardy, 1949, for further discussion of biological oxidations).

During the sequence of events summarized in Figs. 1–3 energy is liberated by oxidation of the foodstuff. It has been postulated with the enzymes of kidney or liver that some 60 per cent of the energy derivable by complete oxidation of carbohydrate can apparently be stored up in the form of so-called "high energy" phosphate bonds (Lipmann, 1941; Hunter and Hixon, 1949). The terminal group of adenosine triphosphate is such a bond and this group can be transferred to synthesize other more stable phosphate compounds or to create active intermediates in the synthesis

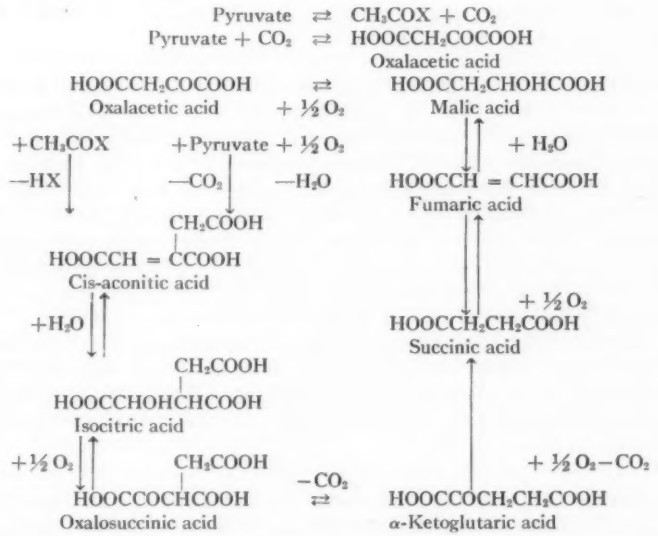

Figure 2. Some possible steps for pyruvate utilization via the tricarboxylic acid cycle.

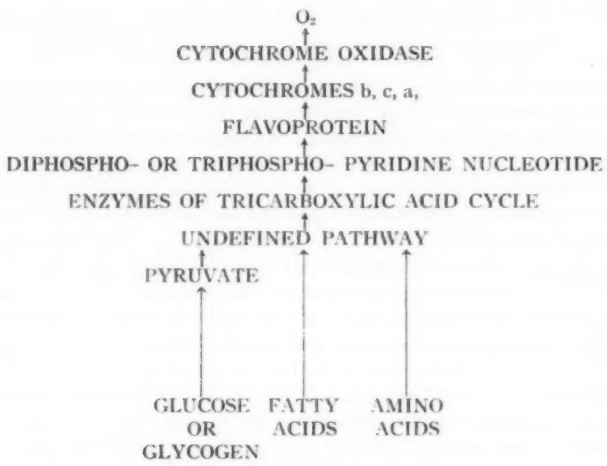

FIGURE 3. Schematic outline of steps by which hydrogens (or electrons) of foodstuffs react with oxygen in animal tissues to form water.

of many cellular constituents. From these facts it is postulated that the major portion of the energy used for synthesis and function must pass through such a mechanism. Most of these "high energy" phosphate bonds are created during the aerobic phase of substrate disposal mentioned above.

None of the enzymes for the anaerobic steps shown in Figure 1 has been studied in Arbacia eggs, nor have specific enzymes for deamination of amino acids or breakdown of fatty acids been looked for.

a. Cytochrome oxidase. Arbacia eggs contain an enzyme which is similar to the cytochrome oxidase of other animal tissues, in that it oxidizes reduced cytochrome c, the cytochrome c concentration for half maximal activity being 4×10^{-6} *M*. The enzyme is reversibly inhibited by carbon monoxide; it is also inhibited by low concentrations of other iron-binding reagents such as cyanide, azide, and sulfide; it is not inhibited by copper-binding reagents such as 8-hydroxyquinoline or diethyldithiocarbamate. This enzyme occurs in nearly equal amounts in unfertilized and fertilized Arbacia eggs, being present in both in a concentration sufficient to account for the respiration of the fertilized eggs even under the maximum respiratory stimulation yet observed (Krahl, Keltch, Neubeck, Clowes, 1941).

Measurements with the Cartesian diver upon centrifugally isolated fractions show the cytochrome oxidase of Arbacia eggs to be associated with the small particulate elements of the cytoplasmic matrix (Hutchens, Kopac, Krahl, 1942) rather than with the particles stainable with Janus green which are customarily referred to as mitochondria (Harvey, 1941).

b. Cytochromes and related compounds. No cytochrome c has been found in Arbacia eggs (Ball and Meyerhof, 1940; Krahl, Keltch, Neubeck, Clowes, 1941). The latter workers used a concentrating procedure which was shown to detect 2

micrograms of added cytochrome c per gram of eggs. This quantity would be equivalent to about 2×10^{-7} *M* in the egg cytoplasm; since the measurements referred to above indicate that the cytochrome oxidase of the egg is fully active only at concentrations above 10^{-5} *M*, and only 10 per cent active at 10^{-6} *M*, considerable doubt arises as to a possible role for cytochrome c in the Arbacia egg.

Ball and Meyerhof found, in the Arbacia egg, iron porphyrin compounds related to the active group of cytochrome c. Cytochromes a and b have not been demonstrable on the basis of spectroscopic observations.

Cytochrome c is plentiful in Arbacia sperm (Ball and Meyerhof, 1940).

c. Flavin-adenine dinucleotide. Flavin-adenine dinucleotide has been extracted from Arbacia eggs and identified by its ability to serve as the prosthetic group of d-amino acid oxidase and by the quantitative absorption spectrum of the lumiflavin obtained from it (Krahl, Keltch, Clowes, 1940). The average content is about 10 micrograms per gram wet eggs, no consistent change with fertilization and development being noted.

d. Diphosphopyridine nucleotide (DPN). DPN has been extracted from the eggs with warm dilute acid, and assayed in terms of its ability to serve as the active group of phosphoglyceraldehyde and glycerophosphate dehydrogenases (Jandorf and Krahl, 1942). The apparent content of DPN in Arbacia eggs at various times after fertilization is, as micrograms per gram wet eggs: unfertilized, 385; fertilized 30 minutes, 345; fertilized 10 hours, 242. With modified extraction procedures, to preserve the acid labile (presumably dihydro-) form of DPN, the total DPN remains constant during the first 10 hours of development at about 300–400 micrograms per gram of eggs.

e. Enzymes of the tricarboxylic acid cycle. The experimental evidence currently available for mammalian tissues indicates that a cycle of reactions (similar to that illustrated in Fig. 2) may provide the mechanism by which the two carbon or three carbon fragments arising from partial breakdown of carbohydrate, amino acids, or fatty acids are converted to carbon dioxide with liberation of energy in a form which cells can use for synthetic reactions or for function. The structure of the initial condensation product arising from pyruvate and oxalacetate is not settled; it may be cis-aconitate, citrate, or possibly some other substance. Pyruvate is oxidized during a sequence of reactions to give three CO_2 molecules per pyruvate. One CO_2 is liberated during formation of the initial six carbon product of condensation, one during the oxidation of oxalosuccinic acid, and one during oxidation of α-ketoglutaric acid to succinic acid. By use of the enzymes from kidney, it has been demonstrated that about 15–20 atoms of inorganic phosphorus are converted into a "high energy" form during oxidation of each pyruvate, most, if not all, during and subsequent to the oxidation of α-ketoglutarate (Ochoa, 1943; Lehninger and Smith, 1949; Hunter and Hixon, 1949).

Efforts to demonstrate the role of the tricarboxylic acid system in Arbacia eggs have so far been confined to determining, first, whether pyruvate can be oxidized by the eggs and, second, whether oxalacetate, α-ketoglutarate, or succinate can be oxidized and, if so, whether "high energy" phosphate is generated thereby.

Oxidation of pyruvate. In most cells both the condensation and the decarboxylation reactions of pyruvate apparently require diphosphothiamine, the biologically active form of vitamin B_1 (Barron and Lyman, 1940).

The diphosphothiamine content of the Arbacia egg was assayed on the basis of the ability of this substance to serve as the active group of yeast decarboxylase. Both Krahl, Jandorf, and Clowes (1942), and Goldinger and Barron (1946) found Arbacia eggs to contain 2–3 micrograms diphosphothiamine per gram wet eggs; this value was not significantly altered by fertilization.

Goldinger and Barron (1946), using a chemical method for pyruvate estimation, found about 70 micrograms of pyruvate per gram dry weight of unfertilized or fertilized Arbacia eggs. They determined the rate of disappearance of pre-formed pyruvate in intact unfertilized and fertilized Arbacia eggs; the rates of pyruvate disappearance at 20° C. were found to be: unfertilized, 66 γ, fertilized, 456 γ, per hour per gram dry weight; these values correspond to 0.2 and 1.3 μM per hour per gram wet weight. Krahl, Jandorf, and Clowes (1942), using a specific but relatively less accurate enzymatic method for pyruvate estimation, determined the rate of disappearance of pyruvate added to cytolysates of unfertilized and fertilized eggs; their values, in μM per gram wet eggs per hour at 20° C., were: unfertilized, 1.1; fertilized, 1.5. It will be noted that the rates for the eggs and for cytolysates are nearly the same after fertilization, but that breakdown of egg structure has raised the rate for cytolysates of unfertilized eggs closer to the fertilized level.

Neither Goldinger and Barron nor Krahl, Jandorf, and Clowes determined the fate of the pyruvate or whether its oxidative removal was coupled with generation of "high energy" phosphate bonds.

Relation of oxidation of oxalacetate, α-ketoglutarate, and succinate to phosphorylation. A cell-free particulate system has been prepared from unfertilized Arbacia eggs which can use substrates of the tricarboxylic acid cycle to effect phosphorylation (Keltch, Strittmatter, Walters, and Clowes, 1949, 1950). Although the effect of these substrates on oxygen consumption is small, owing to a large baseline respiration, phosphorylation is specifically made possible by their oxidation. The phosphorylation is increased from 130 γ per hour in absence of added substrate to 1010 γ with α-ketoglutarate, 910 γ with oxalacetate, and 530 γ with succinate, all values being for a quantity of homogenate obtained from 1 gram wet unfertilized eggs. The ratio: atoms P esterified/atoms oxygen consumed was found to be 1.1 with α-ketoglutarate as substrate.

To prepare a cell-free particulate system capable of oxidizing these substrates, it is necessary to minimize breakdown of the pigment granules during homogenization, as echinochrome apparently inactivates one or more of the catalysts required (Crane and Keltch, 1949). Echinochrome is a substituted naphthoquinone (see below) capable of oxidizing reduced sulfhydryl groups which are known to be essential for oxidation of succinate and other substrates (Hopkins and Morgan, 1938; Barron and Singer, 1943). Earlier unsuccessful attempts to demonstrate oxidation of α-ketoglutarate (Goldinger and Barron, 1946) or succinate (Ball and Meyerhof, 1940; Ballentine, 1940; Goldinger and Barron, 1946) may be due in part to inactivation of the enzymes in egg extracts by echinochrome, and in part to failure of these substances to penetrate into intact eggs.

Homogenates capable of effecting oxidative phosphorylation have been prepared from unfertilized *Strongylocentrotus purpuratus* eggs by Lindberg and Ernster

(1948). Pyocyanine and DPN were added as electron carriers and glucose was added as a supplemental substrate.

f. Oxidation of other substrates. Ballentine (1940b) found, in Arbacia egg cytolysates, no evidence for oxidation of the following substrates: glucose, lactate, α-glycerophosphate, succinate, or butyrate.

Homogenates of other echinoderm eggs have been observed to oxidize a number of substrates in the presence of pyocyanine or other dyes as hydrogen acceptors (see Runnström, 1949, for review). In particular, Lindberg (1943) found that homogenates of Echinocardium eggs could catalyze the reduction of methylene blue by malic, formic, or phosphogluconic acids or by propanediol phosphate. The latter substance was studied because Lindberg isolated a phosphate fraction from sea urchin eggs corresponding in properties to propanediol phosphate. Lindberg (1943) has suggested that part of the energy for development of sea urchin eggs may be derived from oxidation of glucose-6-phosphate over the triphosphopyridine nucleotide-flavoprotein pathway (Warburg and Christian, 1936; Dickens, 1938).

g. Hydrolytic enzymes.

(1) *Adenosine triphosphatase and ATP.* This enzyme, which hydrolyzes adenosine triphosphate (ATP), has been found in eggs of *Strongylocentrotus purpuratus* (Connors and Scheer, 1947) and in *Paracentrotus lividus* (see Runnström, 1949); the ATP-ase activity of the fertilized Strongylocentrotus egg is reported to be higher than that for unfertilized, the amount of inorganic P liberated per hour per mg. egg nitrogen being 10.3 and 4.1 mg., respectively.

A substance similar to, or identical with, adenosine triphosphate was extracted from Arbacia eggs by White and Chambers (1949).

(2) *Phosphatase.* The acid phosphatase activity of the unfertilized Arbacia egg is 3–5 times that of the alkaline phosphatase, and remains constant during the first 40 hours of development; the alkaline phosphatase content remains constant until just before gastrulation and then rises to 10 times that of the unfertilized egg (Mazia, Blumenthal, Benson, 1948). Alkaline phosphatase activity toward the artificial substrate, glycerophosphate, was also studied at various stages of Arbacia development by Krugelis (1947).

(3) *Ribonuclease and desoxyribonuclease.* Evidence for ribonuclease activity in Arbacia eggs has been recorded by Bernstein (1949); Lansing and Rosenthal (1949) found some localization of ribonucleic acid at the surface of Arbacia eggs.

Mazia, Blumenthal, and Benson (1948) studied the distribution of desoxyribonuclease in the Arbacia egg. They found that in the unfertilized egg this enzyme is not restricted to nuclear structures, and that most of the desoxyribonuclease is in the cytoplasm, though no measurable amount of desoxyribonucleic acid is found there. During the first 40 hours of development after fertilization, there is no increase in the total desoxyribonuclease content of the egg, but the fraction of the enzyme which is non-sedimentable at 20,000 g. declines as development proceeds, being about 10–20 per cent of the total activity at 40 hours.

(4) *Peptidases.* Holter and co-workers (1936, 1938) studied the peptidase activity toward alanylglycine of unfertilized Arbacia eggs and fractions prepared from them by centrifuging. Holter found that the peptidase is localized in the hyaline ground substance (matrix) of the egg cytoplasm and not bound to granular matter.

Doyle (1938) found the peptidase content of *Psammechinus miliaris* to be the same before and after fertilization, in agreement with the experiments of Holter (1936) upon Echinarachnius.

Lundblad (1949) demonstrated proteolytic activity toward gelatin in eggs and sperm of *Arbacia lixula* and sperm of *Paracentrotus lividus*.

h. Catalase. Catalase activity was found in eggs and egg fragments of Psammechinus by Doyle (1938). Fertilized eggs with intact membranes showed lower catalase activities than unfertilized eggs. Fertilized eggs with membranes destroyed showed the same catalase activity as unfertilized eggs. (For earlier observations on catalase in Arbacia eggs, see Harvey review, 1932.)

2. Metabolic Intermediates. During the first eight hours of development, the synthetic activities of the sea urchin egg consist chiefly in rearranging the nuclear and cytoplasmic components, with little or no increase in the total organic mass of the egg (Gray, 1927; E. B. Harvey, 1949). The question of what foodstuffs are available and used to provide energy for these synthetic activities according to the reactions illustrated in Figures 1–3 has been given only preliminary study in *Arbacia punctulata* eggs, and has received somewhat more attention in European sea urchins (see Örström and Lindberg, 1940; and Lindberg, 1946).

a. Total acid-hydrolyzable polysaccharide. This was reported to be equivalent to 50 mg. glucose per gram Arbacia egg protein by Perlzweig and Barron (1928), and 110 mg. glucose per gram egg protein by Hutchens, Keltch, Krahl, and Clowes (1942). The discrepancy was attributed by the latter authors, in part, to differences in the time of the season at which the eggs were collected and used, as they found that the carbohydrate values were higher from stored than from recently collected urchins. Confirming this suggestion, it was later found that the nitrogen content of the Arbacia egg tends to fall during the latter part of the season, especially if urchins are stored for several days or weeks in indoor aquaria (Crane, 1947). This nitrogen loss tends to elevate analytical values which are based on egg nitrogen or protein content.

Of the acid-hydrolyzable polysaccharide, only a negligible amount comes from the jelly (Hutchens, Keltch, Krahl, and Clowes, 1942).

No free reducing sugar was found by either group of investigators.

b. Glycogen. Of the 110 mg. total acid-hydrolyzable polysaccharide per gram egg protein found by Hutchens, Keltch, *et al.* (1942), 46 per cent was isolated as an alkali-stable, alcohol-precipitable polysaccharide. This substance corresponds to typical animal glycogen in optical rotation, yield of glucose upon hydrolysis, and reaction with iodine. This glycogen content is of the same order as that (50–80 mg. per gram egg protein) obtained for Arbacia by Blanchard (1935) and for the European sea urchins *Paracentrotus lividus* and *Echinus esculentus* (see Örström and Lindberg, 1940, for a summary of the total carbohydrate and glycogen contents of these eggs).

c. Other possible metabolic intermediates. Other metabolic intermediates indicated in Figures 1 and 2 have not been looked for in Arbacia eggs, but the phos-

phate fractions of European sea urchins have been studied by Zielinski (1939) and Lindberg (1943). In Echinocardium eggs, the latter reported that there are only three principal forms of phosphate; 53 per cent of the total being inorganic orthophosphate, 31 per cent adenosine triphosphate, and 16 per cent propanediolphosphate.

d. Fats. The total fat in 10^6 unfertilized eggs is 5.65 mg., of which 7.5 per cent is sterol and 38 per cent phospholipid. After fertilization, the total decreases up to 8 hours, increases to 20 hours, and then declines again up to 40 hours (Hayes, 1938).

Parpart (1941) found the total lipid to be 5.4 per cent of the whole egg, or 26.9 per cent of egg solids; 77 per cent of the lipid appeared to be bound to protein. Neither the total lipid nor lipid bound to protein was found to change during the first 5 hours of development.

Navez and E. B. Harvey (1935), Navez (1939), and Navez and DuBois (1940) have studied the unsaturated fatty acid fraction of Arbacia eggs, some component of which acts as a catalyst in oxidation of the Nadi reagent, thus simulating the enzyme indophenol oxidase.

e. Foodstuffs consumed and products formed during cleavage. Hutchens, Keltch, Krahl, and Clowes (1942) measured the glucose used, and the lactic acid, carbon dioxide, and ammonia formed by the fertilized Arbacia egg during the first few hours after fertilization. Their observations are as follows: (1) little or no lactic acid is formed; (2) the glucose used is, in 5 of 6 experiments, not adequate to account for the oxygen consumed; (3) the oxygen required for protein oxidation (calculated on basis of ammonia produced) is sometimes greater, sometimes less, than the oxygen consumption not accounted for by carbohydrate oxidation. For the periods in which the total oxygen consumption could not be accounted for by the sum of apparent carbohydrate and protein oxidation, the respiratory quotients are low, suggesting that fat may be oxidized.

Perlzweig and Barron (1928) had previously measured the lactic acid content before and immediately after fertilization.

Zielinski (1939) and Örström and Lindberg (1940) found a decrease in carbohydrate content of various sea urchins to occur upon fertilization, but they did not study the period 0.5–3 hours after fertilization, so that their results cannot be directly compared with those of Hutchens, Keltch, Krahl, and Clowes (1942).

3. Phosphorus and Nucleic Acid Metabolism in Arbacia Eggs. Determinations of the total phosphorus of the Arbacia egg and its partition are given in Table II.

Measurements of the rate of uptake of inorganic P^{32} from sea water by Arbacia were made by Abelson (1947, 1948). He found the total P^{32} uptake by fertilized eggs in a given time to be 40 times that of the unfertilized eggs; the amount taken up by fertilized eggs at 10° C. was only one-seventh that at 23° C. Of the total P^{32} taken up by fertilized eggs, 97 per cent appeared in the acid-soluble fraction. Of this acid-soluble fraction, 16 per cent was inorganic phosphate, 41 per cent adenosine triphosphate and diphosphate, 41 per cent barium-soluble, and 2 per cent unaccounted for. These measurements were made using carriers for inorganic

TABLE II

Analyses of the phosphorus fractions of Arbacia eggs, milligrams P per gram wet weight egg or embryo (modified from Villee, Lowens, Gordon, Leonard and Rich, 1949)

Investigator	Stage	Acid soluble P	Phospho-lipid P	Acid insoluble P	DNA P	RNA P	Phospho-protein P	Total P
Crane (1947)	unfert. eggs	1.3	1.3	0.86	0.03			3.2[1]
Schmidt, Hecht, and Thannhauser (1948)	unfert. eggs	—	1.5	1.0	0.04	0.86	0.11	4.1
Villee *et al.* (1949)	3 hrs. embryos	1.4	—	0.86	0.05	0.74	0.06	—

[1] Analyses for individual fractions were carried out on separate batches of eggs, and the original values were based on egg nitrogen content. Later, Crane found that the nitrogen content of various batches of eggs decreased with increasing periods of storage of the urchins. For this reason, the total P is not equal to the sum of the individual fractions.

phosphate and ATP, and were recorded with some reserve because of interference of echinochrome with the phosphate fractionation.

The rates at which inorganic P^{32} enters the various organic fractions of the fertilized Arbacia egg were determined by Villee, Lowens, Gordon, Leonard, and Rich (1949). After 6 hours of development, the relative quantities of P^{32} in the various fractions were (expressed as counts per minute per mg. $P \times 10^3$): acid soluble—P, 20; RNA—P, 61; acid insoluble—P, 75; DNA—P, 109; phosphoprotein—P, 135. On the basis of the fact that P^{32} enters the DNA more rapidly than the RNA fraction, they conclude that the DNA of the fertilized egg is not formed from the RNA of the unfertilized egg, as had been suggested for eggs of *Paracentrotus lividus* by Brachet (1947).

The P^{32} uptake by other sea urchin eggs has also been measured. Lindberg (1949) has determined the rate at which P^{32} is incorporated into the acid-soluble, acid-labile fraction (presumed by Lindberg to be adenosine triphosphate) of Psammechinus eggs. In the unfertilized eggs, the initial rate of P^{32} uptake into this fraction is low, and a steady state is reached within 5 minutes after exposure to the P^{32}. In the fertilized egg, the rate of P^{32} incorporation into this fraction is much higher, and is sustained for at least 60 minutes after fertilization.

Measurements of P^{32} uptake have been made upon *Strongylocentrotus* (*purpuratus* and *franciscanus*) by Brooks and Chambers (1948), Chambers and White (1949), and upon *Lytechinus pictus* by Chambers, Whiteley, Chambers, and Brooks (1948).

Schmidt, Hecht, and Thannhauser (1948) reported that the unfertilized *Arbacia punctulata* egg contains 20×10^{-5} micrograms ribonucleic acid—P(RNA–P) and 1×10^{-5} micrograms desoxynucleic acid—P(DNA–P) per egg, i.e. 0.86 and 0.04 mg. per wet gram of eggs, respectively. During the first 24 hours of development, the amount of ribonucleic acid per embryo remains virtually unchanged, but the desoxynucleic acid steadily increases until, at the end of this period, the desoxyribosenucleic acid content per embryo is 10–15 times that of the unfertilized egg.

Blanchard (1935) had previously isolated 1.08 grams of a relatively pure sample of desoxynucleic acid from 4820 grams of wet unfertilized Arbacia eggs; he also isolated a small amount of ribosenucleic acid.

4. Calcium Metabolism. Heilbrunn, Mazia, and Steinbach (1934) and Mazia (1937) have found that the proportion of bound calcium ion in Arbacia eggs decreases by about 15 per cent on fertilization, the total calcium remaining constant. Mazia calculated that the free calcium of the unfertilized eggs is about 0.0005 molar, increasing by 0.001 molar upon fertilization. The relation of calcium ion to activation processes has been discussed by Heilbrunn (1943).

Changes in free calcium in other echinoderm eggs have been measured by Örström and Örström (1942) and Monroy-Oddo (1946). Hultin (1949) found that calcium ion increased both oxygen uptake and anaerobic acid formation in homogenates of Psammechinus eggs.

5. Echinochrome. The precise function of echinochrome in metabolism of the Arbacia egg has yet to be defined. The observations of Keltch and co-workers (1949, 1950), recorded above, suggest that echinochrome, when it leaves the pigment granules and gains access to the egg cytoplasm, may inactivate enzymes which contain sulfhydryl groups.

Crystalline echinochrome was isolated from the eggs and tests of *Arbacia punctulata* by Ball (1936). The pigment is capable of reversible oxidation-reduction and has a potential, E_0' at pH 7 and 30° C., of -0.221 V. and an E_0 of $+0.1995$ V. (Cannan, 1927). The structure of echinochrome from *Arbacia punctulata* eggs has not been determined, but that from *Arbacia lixula* eggs, which is presumed to be similar to that from *Arbacia punctulata*, has been shown to be (in the reduced form) 1,3,4,5,6,7,8-heptahydroxy-2-ethylnaphthalene (Kuhn and Wallenfels, 1939).

The spectrophotometric properties for echinochrome from the eggs are the same as for that from the amoebocyte and test of *Arbacia punctulata* (Ball and Cooper, 1949). Echinochrome has an acid group of pK′ 6.38 at 26° C. The acid form has 3 absorption peaks at 255, 335, 475 mμ, with molecular absorption coefficients of 1.93×10^4, 0.87×10^4, 0.65×10^4, respectively. The three peaks for the ionized form are centered at 275, 400, and 475 mμ with molecular absorption coefficients of 2.11×10^4, 0.88×10^4, and 1.12×10^4, respectively. The eggs contain on the average 0.58 gram echinochrome ($C_{12}H_{10}O_7$) per 100 cc. of eggs packed by centrifuging.

E. Penetration of Chemical Agents into Arbacia Eggs

The effects of many weak acids and bases upon Arbacia eggs are strongly dependent upon the pH of the sea water medium in which the eggs are suspended.

Detailed analyses of the relation of pH to intensity of physiological effect in Arbacia eggs have been made for carbon dioxide (Smith and Clowes, 1924; Haywood and Root, 1930, 1932); for substituted phenols (Krahl and Clowes, 1938; Hutchens, Krahl, and Clowes, 1939); for barbiturates (Krahl, 1940; Clowes, Keltch, and Krahl, 1940); and for local anesthetics (Krahl, Keltch, and Clowes, 1940).

The total concentration of each phenol, barbiturate, or local anesthetic required to produce 50 per cent inhibition of cleavage was measured at each of several extracellular pH values, and the concentration of undissociated molecules and ionized molecules was calculated in the conventional way from the equation:

$$pH = pK'_a + \log \frac{\text{anion concentration}}{\text{undissociated molecule concentration}}$$

for the weak acids and the analogous equation:

$$pH = pK'_{water} - pK'_b - \log \frac{\text{cation concentration}}{\text{undissociated molecule concentration}}$$

for bases, where pK'_a, pK'_b, pK'_w are the negative logarithms of the dissociation constants of the acid, the base, or water, respectively. It was found, with any one agent, that the concentration of *undissociated molecules* required to produce 50 per cent inhibitition of the rate of cleavage is the same at various extracellular pH values, whereas neither the total concentration nor the ion concentration remains constant as the pH is varied. An example is shown in Table III. These observations were

TABLE III

Concentration of undissociated substituted phenol molecules required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at pH 6.6 and pH 8.1 in glycyl glycine buffer (Krahl and Clowes, 1938a)

Temperature 20° C.

Substituted phenol	pK'	Total concentration		Extracellular molecule concentration	
		pH 6.6	pH 8.1	pH 6.6	pH 8.1
		moles per l.	moles per l.	moles per l.	moles per l.
2,4-dinitrophenol	4.1	3.4×10^{-6}	6.3×10^{-5}	1.1×10^{-8}	0.6×10^{-8}
4,6-dinitro-o-cresol	4.4	8.0×10^{-7}	1.5×10^{-5}	5.1×10^{-9}	3.8×10^{-9}
2,4,5-trichlorophenol	6.9	1.7×10^{-6}	1.5×10^{-5}	1.1×10^{-6}	1.1×10^{-6}
2,4-dichlorophenol	7.7	3.2×10^{-6}	1.1×10^{-4}	3.0×10^{-6}	3.1×10^{-5}
m-nitrophenol	8.3	1.7×10^{-4}	3.2×10^{-4}	1.7×10^{-4}	2.0×10^{-4}

interpreted to indicate that these weak acids and bases penetrate Arbacia eggs principally, and in most cases entirely, in the form of undissociated molecules. In the course of these same studies, an attempt was made to identify the form in which these agents act within the cell.

Tyler and Horowitz (1937) found that the concentration of the anion of each of 8 substituted phenols required to produce 90 per cent inhibition of cleavage of eggs of *Strongylocentrotus purpuratus* is the same, from which they concluded that the active agent in blocking cleavage was the anion. In contrast, Krahl and Clowes (1938), using a more complete series of 30 substituted phenols, upon Arbacia eggs, found that the calculated anion concentrations required to produce 50 per cent inhibition of cleavage vary from 0.1×10^{-6} *M* for 2,4-dinitro-o-cyclohexylphenol up to 192×10^{-6} *M* for 2,4-dinitro-o-isopropylphenol. The measured values from which these calculations were made are given in Table IV. Thus, the attrac-

tive hypothesis of Tyler and Horowitz is not supported by more extensive data upon Arbacia. Krahl and Clowes (1938) and Hutchens, Krahl, and Clowes (1939), after studying effects of these agents at varying intracellular and extracellular pH values, drew the tentative conclusion that the undissociated molecule was the form active in inhibiting cleavage, while the anion might be the active agent in stimulating oxygen consumption (see below).

Though not studied in detail, the variation in effects of other weak acids with pH is qualitatively like that of the agents mentioned above. The physiological effects upon Arbacia eggs of hydrogen cyanide (pK′ 9.2), hydrazoic acid (pK′ 4.7), hydrogen sulfide (pK′ 7.0), and iodoacetic acid (pK′ 3.1) are more marked in acid

TABLE IV

Effects of substituted phenols on oxygen consumption and cleavage of fertilized eggs of Arbacia punctulata at pH 7.5 and 20° C.

(From Clowes and Krahl, 1936; Krahl and Clowes, 1936, 1938a)

Substituted phenol	pK′ at 25° C.	Oxygen consumption, maximum rate, as per cent of normal[1]	Cleavage, total conc. to give 50 per cent inhibition
			moles per l. × 10^5
1. 2,4-dinitro-o-cyclohexylphenol	4.2	—	0.6
2. 2,4-dinitro-ar-tetrahydro-α-naphthol	4.5	320	1
3. 4,6-dinitro-2-(1,1-dimethylhexyl)-phenol	—	260	1
4. 2,4-dinitrothymol	4.1	no stimulation	1
5. 4-nitrocarvacrol	4.2	—	2
6. 2,4,6-triiodophenol	6.6	180	3
7. 4,6-dinitro-o-cresol	4.4	300	4
8. 2,4-dinitro-α-naphthol	3.6	230	6
9. 2,4,5-trichlorophenol	6.9	250	10
10. 2,4-dinitro-o-phenylphenol	3.9	—	10
11. 2,4,6-tribromophenol	6.0	230	16
12. 2,4-dinitrophenol	4.1	290	23
13. 4,6-dichloro-o-cresol	7.7	190	25
14. 2,6-dibromo-4-nitrophenol	3.7	240	25
15. 4,6-dinitrocarvacrol	4.5	360	46
16. 2,6-dichloro-4-nitrophenol	3.7	230	55
17. 2,6-dinitrophenol	3.7	270	55
18. 4,6-dibromo-o-cresol	7.6	230	98
19. 2,4,6-trichlorophenol	6.2	220	98
20. 2,4-dichlorophenol	7.7	230	100
21. p-nitrophenol	7.2	325	100
22. 2-hydroxy-5-chlorobenzaldehyde	7.4	—	125
23. 2,4-dibromophenol	7.8	270	138
24. 2-nitro-4-chlorophenol	3.5	250	184
25. 2-hydroxy-5-bromobenzaldehyde	7.4	—	250
26. m-nitrophenol	8.3	230	314
27. 2,6-dinitro-4-chlorophenol	3.5	210	400
28. 2,6-dibromophenol	6.6	120	900
29. 2,4-dinitro-o-isopropylphenol	3.0	—	1024
30. 2,4,6-trinitrophenol	0.7	no stimulation	6900
31. o-nitrophenol	7.2	no stimulation	22,600

[1] The concentrations required to produce maximal stimulation of oxygen consumption are about 0.5-1 times those required to produce 50 per cent inhibition of cleavage.

than in neutral or alkaline solutions (Clowes and Krahl, 1940). The sodium or potassium salts of such weak acids dissolve to give alkaline solutions which must be neutralized and buffered in the physiological range.

These principles must be kept in mind in comparing measurements of various investigators who have, in many cases, taken insufficient account of the effect of pH upon penetration of the agent under study.

F. Effects of Chemical Agents upon Oxygen Consumption, Cleavage, and Metabolic Enzymes

Effects of chemical agents upon Arbacia eggs have been studied from a number of points of view. The two principal objectives, with examples of agents used to approach them, are: First, elucidation of the processes by which energy is provided for development of the egg—low oxygen tension, cyanide, carbon monoxide, azide, sulfide, inhibitors of copper catalysts, iodoacetate, substituted phenols, urethane, naphthoquinones, and dyes; second, analysis of the biochemical mechanism of action of physiologically active agents, including mechanism by which agents penetrate cell surfaces—substituted phenols, barbiturates, local anesthetics, xanthines, penicillins, sulfonamides, colchicine, podophyllotoxin, and a number of other agents.

1. Low Oxygen Tension. The problem of requirement of oxygen for cleavage of sea urchin eggs was first posed specifically by J. Loeb (1895); he found that Arbacia eggs could not undergo sustained cleavage when oxygen tension is reduced by replacement of air with hydrogen. More detailed study showed that cleavage is completely suppressed at a level of 0.4 per cent oxygen—99.6 per cent nitrogen, where the oxygen consumption is 20–30 per cent of normal (Amberson, 1928; Clowes and Krahl, 1940).

Cytological observations by E. B. Harvey (1930) and Fry (1937) indicate that detailed metabolic activities are blocked either immediately or within a few minutes after the eggs are brought under anaerobic conditions. This indicates that the Arbacia egg has neither a reserve energy source nor a mechanism for energy liberation upon which it can support cleavage when oxygen is lacking.

2. Cyanide, Carbon Monoxide, Azide, Sulfide, Substances forming Complexes with Copper. The effects of each of these agents have been tested upon egg respiration and cleavage, and upon cytochrome oxidase from the eggs.

Cyanide—In the unfertilized egg, about 50 per cent of the oxygen consumption is cyanide sensitive (Clowes and Krahl, 1934; Robbie, 1946).

Effects of cyanide upon oxygen consumption and cleavage in fertilized Arbacia eggs have been measured by a number of investigators (Table V). For other data which are not in a form adaptable to tabulation see Brooks (1933, 1941), Brinley (1930), Blumenthal (1930), Barron and Hamburger (1932), Shapiro (1940), Krahl and Clowes (1940).

Loeb (1906) observed that fertilized *Strongylocentrotus purpuratus* eggs would not divide in solutions of potassium cyanide. Apparently the first simultaneous measurements on both respiration and cleavage in presence of cyanide were made by Warburg (1910) upon eggs of *Strongylocentrotus lividus;* cleavage was blocked at a concentration of 0.0001 *M* cyanide, in which oxygen consumption was reduced

to about 20 per cent of normal. These observations by Warburg were the first in a long series which led eventually to his characterization of the *Atmungsferment* as a metalloprotein and to the isolation in pure form of the yellow enzymes (flavoproteins) and of triphosphopyridine nucleotide.

Cleavage of the fertilized Arbacia egg is blocked at 20° C. when oxygen consumption is reduced by cyanide to about 35 per cent of normal (Clowes and Krahl, 1940; Robbie, 1946), but the concentration required to produce this effect cannot be precisely stated, owing to the dependence of this concentration upon the conditions of measurement. Potassium cyanide, at pH values in the physiological range,

TABLE V

Effect of cyanide upon oxygen consumption and cleavage of sea urchin eggs

Investigator	Unfert. (U) or fert. (F)	Temp. °C.	pH of medium	Oxygen consumption			Cleavage, concentration required for 100 per cent inhibition
				Inhibition at cyanide conc. required for 100 per cent inhib. of cleavage	Degree of maximal inhibition	Conc. required for maximal inhibition	
				per cent of control	per cent of control	moles per l.	moles per l.
Warburg (1910)[1]	F	—	—	80	80	1×10^{-4}	1×10^{-4}
Loeb and Wasteneys (1911)[2]	F	20	—	75	—	—	4×10^{-5}
Loeb and Wasteneys (1913)[2]	F	14	—	67	—	—	3×10^{-5}
Barron and Hamburger (1932)[3]	F	24	8.2	—	100	1×10^{-3}	—
Clowes and Krahl (1934b)[3]	U	21	8.2	—	50	1.3×10^{-3}	—
Korr (1937)[3,4]	F	15	—	—	39	2.5×10^{-3}	—
		20	—	—	49	2.5×10^{-3}	—
		25	—	—	57	2.5×10^{-3}	—
Lindahl (1940)[5]	U	19–22	—	—	43	1×10^{-3}	—
	F	19–22	—	—	88	1×10^{-3}	—
Clowes and Krahl (1940)[3]	F	20	7.9	65	75	1×10^{-3}	1.6×10^{-4}
		24	7.9	—	84	1×10^{-3}	—
Robbie (1946)[3]	U	20	8.0	—	57	1×10^{-4}	—
	F	20	8.0	65	82	1×10^{-4}	1.5×10^{-5}

[1] *Strongylocentrotus lividus.*
[2] *Strongylocentrotus purpuratus.*
[3] *Arbacia punctulata.*
[4] Values are given for temperature from 11–26°. Only one cyanide concentration was used (2.5×10^{-3}M); this was stated to be higher than that required for maximum inhibition.
[5] *Paracentrotus.* Values are also given for oxygen tensions other than that in air.

is in equilibrium with HCN, which is volatile and distills into the alkali used to absorb CO_2 in respiratory measurements by the Warburg method. Robbie (1946) has given detailed procedures for minimizing this difficulty. The opposite situation obtains in the older measurements by the Winkler method where respiratory CO_2 was allowed to accumulate; here the pH tends to fall and make the required concentration of cyanide lower than it would be at pH 8. All methods agree in indicating that the critical concentration of cyanide to block cleavage of Arbacia eggs is about 10^{-5}–10^{-4} *M* in unbuffered sea water.

As the cyanide concentration is increased, the oxygen consumption passes through a minimum; at the maximally inhibiting cyanide concentration, the oxygen consumption of the fertilized eggs is reduced by some 75–80 per cent (Warburg, 1910; Clowes and Krahl, 1940; Robbie, 1946). The fact that Korr (1937) observed a smaller degree of inhibition (49 per cent at 20° C.) may be due to his use of a concentration of cyanide above that which gives maximum inhibition. Lindahl (1940) found the inhibition by 0.001 *M* cyanide to be greater at low oxygen tensions than at 100 per cent oxygen, and Robbie (1946) likewise found a greater inhibition by 10^{-4} *M* cyanide as oxygen tension was lowered.

Cytochrome oxidase activity of Arbacia eggs is strongly inhibited by low concentrations of sodium cyanide, 50 per cent at about 3×10^{-6} *M*, and 100 per cent at about 2×10^{-4} *M* under the conditions of these experiments (pH 6.9). The degree of inhibition by a given concentration of cyanide is not greatly altered by variation in concentration of cytochrome c over the range from 0.34×10^{-5} *M* to 1.36×10^{-5} *M* nor by use of p-phenylene diamine instead of hydroquinone as substrate (Krahl, Keltch, Neubeck, Clowes, 1941).

Carbon monoxide—Carbon monoxide has the specific property of entering into catalytically inactive light-reversible combination with the ferrous iron of certain iron-porphyrin compounds (Warburg, 1926; Keilin, 1930). Its effects on oxygen uptake by isolated tissues are considered at present to be entirely attributable to this mechanism; an inhibition by carbon monoxide is thus more strongly indicative of dependence of respiration upon iron-porphyrin compounds than inhibition by cyanide, azide, or sulfide, which can combine readily with other metal-containing pigments than ferric porphyrin compounds, and which can also act as reducing agents.

With $CO-O_2$ mixtures in the dark, cell division is approximately 50 per cent inhibited at 2 per cent oxygen—98 per cent CO; completely and reversibly inhibited at 0.7 per cent oxygen – 99.3 per cent CO, at which point oxygen consumption is about 30 per cent of that in the control (Clowes and Krahl, 1940). The complete cell division block occurs at the same level of oxygen consumption as when simple oxygen lack is the division inhibiting agency; hence carbon monoxide (in contrast to azide; see below) appears to have no effect on cleavage other than that arising from its effect on oxygen consumption.

Both the partial inhibition of oxygen consumption and the partial suppression of cleavage by mixtures of 1.6 per cent O_2—98.4 per cent CO, or 2.1 per cent O_2—97.9 per cent CO, are reversed by light (Clowes and Krahl, 1940).

Carbon monoxide produces a light-reversible inhibition of the cytochrome oxidase from Arbacia eggs (Krahl, Keltch, Neubeck, Clowes, 1941).

The inhibition constant, $K = \frac{n}{1-n} \cdot \frac{pCO}{pO_2}$, where n is the per cent inhibition in the dark and pCO and pO_2 are the respective carbon monoxide and oxygen partial pressures, was calculated. For the cell-free cytochrome oxidase system from Arbacia eggs the value is about 3.1–3.2, at either 0.34×10^{-5} *M* or 1.36×10^{-5} *M* cytochrome c and 0.02 *M* hydroquinone as oxidizable substrate; it is 4.5 at 1.36×10^{-5} *M* cytochrome c and 0.001 *M* hydroquinone. For the respiration of the intact fertilized Arbacia egg the value of this inhibition constant is about 60, as compared to 5 for yeast saturated with glucose as substrate (Warburg, 1927) and 21 for fertilized *Paracentrotus lividus* eggs (Runnström, 1930).

The light-reversible carbon monoxide inhibition of respiration and cleavage is perhaps the best, if not the sole, evidence for participation of an iron-porphyrin catalyst related to cytochrome oxidase in the sequence of oxidative reactions which yield energy to support cleavage. But the precise nature of this catalyst must be settled by further investigation, there being two anomalies to be explained: first, the very low concentration in the egg of cytochrome c, the usual substrate for the cytochrome oxidase system; and second, the greater apparent affinity of CO for cell-free cytochrome oxidase than for the CO sensitive enzyme of the intact egg. The CO inhibition constant may provide a valuable criterion for the identification of the substance which is actually oxidized by the cytochrome oxidase in the egg.

No inhibition of oxygen consumption of unfertilized Paracentrotus or Arbacia eggs by carbon monoxide can be demonstrated (Runnström, 1930; Brooks, 1941). Brooks (1941) reported that unfertilized Arbacia eggs consume carbon monoxide at the same rate, and fertilized eggs at one-third the rate, that they consume oxygen. Any such carbon monoxide consumption must be taken into account in a quantitative reexamination of the carbon monoxide inhibition constants, but it should be noted that correction for CO consumption would make the inhibition constant for the fertilized eggs even higher than that observed.

Azide—Only 50 per cent of the respiration of fertilized Arbacia eggs is insensitive to azide; moreover, 50 per cent inhibition of cleavage occurred at an azide concentration (3×10^{-3} *M* at pH 7.9) inhibiting respiration by only about 10 per cent; complete and reversible inhibition of cell division occurred at an azide concentration (5×10^{-3} *M*) inhibiting respiration by 50 per cent (Clowes and Krahl, 1940). When considered in conjunction with the effects of oxygen lack, cyanide, and carbon monoxide mentioned above, these measurements indicate that azide interferes with cleavage by a mechanism other than by simple reduction of oxygen consumption. A possible clue to the nature of this action is found in the observation that azide can block "high energy" phosphate transfer in a cell-free system (Loomis and Lipmann, 1949) and in yeast (Reiner and Spiegelman, 1947). Cytochrome oxidase of Arbacia is 50 per cent inhibited by 6.4×10^{-5} *M* azide at pH 6.8 and 20° C. (Krahl, Keltch, Neubeck, Clowes, 1941).

Sulfide—The effect of sodium sulfide on respiration and cell division of fertilized Arbacia eggs was determined in flasks of the Dixon and Keilin type, in which alkali is not present during the period of oxygen uptake, and distillation of volatile acids such as H_2S from the medium during the period of measurement does not occur. Cleavage is 50 per cent inhibited at a sulfide concentration (10^{-4} *M*) inhibiting respiration by only 10 per cent. Complete but not reversible inhibition of cleavage

occurs at a sulfide concentration (2×10^{-4} *M*) inhibiting respiration by about 50 per cent. Thus, the hydrogen sulfide-sulfide system, like the hydrazoic acid-azide system, appears to have some effect on cleavage which is not attributable merely to its effect on oxygen uptake (Krahl, Keltch, Neubeck, Clowes, 1941).

Cytochrome oxidase of Arbacia eggs is also inhibited by sodium sulfide, complete suppression of activity being produced at approximately 1×10^{-3} *M* sodium sulfide at pH 6.9. But at higher concentrations, sulfide is rapidly oxidized by the egg cytochrome oxidase preparation, so that the precise inhibition-concentration relationships are difficult to establish.

Substances forming copper complexes—In view of the suggestion of Keilin and Hartree (1938) that cytochrome oxidase may be a copper compound, effects of a number of inhibitors of copper catalysts on egg respiration and cleavage and on Arbacia cytochrome oxidase were explored. Sodium diethyldithiocarbamate and 8-hydroxyquinoline did not inhibit either oxygen consumption of fertilized eggs or the cytochrome oxidase therefrom up to concentrations of 5×10^{-3} *M*. Potassium dithiooxalate (10^{-3} *M*), diphenylthiocarbazone (10^{-4} *M*), isonitrosoacetophenone (2×10^{-3} *M*), or 8-hydroxyquinoline (5×10^{-5} *M*) did not inhibit cleavage of the fertilized eggs. Diethyldithiocarbamate blocked cleavage irreversibly at a concentration of 3×10^{-5} *M* (Table VIII); at higher concentrations, cleavage proceeded through the 16 cell stage, but the eggs were irreversibly injured, as they did not survive to swimming forms when returned to sea water at this point. This anomalous effect of the dithiocarbamates may be exerted by a mechanism other than combination with copper (Clowes and Krahl, 1940; Krahl, Keltch, Neubeck, Clowes, 1941).

3. Substituted Phenols and Related Substances. The effects of these substances upon oxygen consumption and cleavage of Arbacia eggs have been intensively studied by Krahl, Clowes, Keltch, and their collaborators (1936, 1938, 1940, 1949). As the effects of these substances upon enzyme systems have proved to be relatively specific (see below), their effects upon Arbacia eggs may be given in some detail.

a. Effects on eggs. The typical effect is illustrated by the behavior of 4,6-dinitro-o-cresol. As the concentration is increased, oxygen consumption is increased to a maximum at a concentration of about 4×10^{-6} *M*. Above this concentration, oxygen consumption drops off, and a reversible complete block to cleavage sets in at about 2×10^{-5} *M*, at which point oxygen consumption is still above normal (Clowes and Krahl, 1934a, 1936). Ellis (1933) had previously noted that first cleavage of fertilized eggs of *Urechis caupo* was delayed by 0.001 per cent 2,4-dinitrophenol.

These effects are not dependent on reversible oxidation-reduction of the nitro groups, as halogenated phenols act like nitrophenols with respect to both respiration and cleavage of fertilized Arbacia eggs (Krahl and Clowes, 1936).

The concentrations required to stimulate respiration and to block cleavage of fertilized Arbacia eggs are summarized for 30 substituted phenols in Table IV. From these data it can be seen that these agents vary substantially in their ability to inhibit cleavage, whether total concentration, undissociated molecule concentration, or anion concentration be used as the index of effectiveness (Krahl and

Clowes, 1938). In general, the concentration required to produce 50 per cent inhibition of cleavage is equal to, or slightly larger than, that which produces optimum respiratory stimulation.

The active substituted phenols also stimulate oxygen consumption of unfertilized Arbacia eggs (Clowes and Krahl, 1934b, 1936) and of artificially fertilized eggs (Keltch and Clowes, 1947). In the latter case the optimum level of oxygen uptake attained is approximately the same as that produced by the same agent upon sperm-fertilized eggs; the cleavage of the artificially fertilized eggs is blocked at approximately the same concentrations of 4,6-dinitrocresol or 2,4,5-trichlorophenol as those required for sperm-fertilized eggs (Keltch, Walters and Clowes, 1947).

Stimulation of oxygen consumption of unfertilized eggs by substituted phenols has not resulted in artificial parthenogenesis, as measured by appearance of a fertilization membrane.

Inhibition by nitrophenols of sperm fertilization requires concentrations much higher than those which block cleavage (Clowes, Krahl and Keltch, 1937).

Observations upon related compounds which do not exhibit the typical substituted phenol effect upon respiration and cleavage of Arbacia are summarized by Clowes and Krahl (1936).

For combined actions of substituted phenols, carbon monoxide, and other agents, see Krahl and Clowes (1940).

Cytological studies by Fry (1935) showed that eggs first exposed to an active substituted phenol at the metaphase, anaphase, telophase, or resting stages apparently tended to cease development in the subsequent prophase. Fertilized eggs suitably exposed to cyanide or x-radiation also tended to accumulate in the subsequent prophase. At first sight, this suggested that all these agents had some especially intense action at prophase. To attempt to settle this point, Fry and Krahl (1936) constructed a mitotic time schedule which would indicate the proportion of eggs in all mitotic stages at various times after fertilization. This enabled estimates of the average *normal* time spent by any given egg in a given mitotic stage to be made, and showed that this time was much longer for prophase than for any other phase, as was well known by qualitative observations upon the living egg. When the results with substituted phenols were quantitatively compared with this mitotic time schedule, it was found that the time spent by the inhibited eggs in prophase was perhaps *slightly* greater (relative to normal) than for other phases, but that the accumulation of prophase figures was principally due to the long duration of prophase and the resultant greater chance of trapping eggs at this stage. These considerations should be kept in mind in postulating a specific effect of any agent upon a specific stage in mitosis.

A more detailed general discussion of the substituted phenols has been given by Clowes (1948).

b. Effects on enzymes. The mechanism of the two phases of the substituted phenol action on Arbacia eggs, namely, the stimulation of oxygen consumption on the one hand and the inhibition of cleavage on the other, has received intensive study.

The analysis of the first phase of the effect was long hampered by the fact that the oxidative stimulation was attainable only with intact cells. No augmentation of activity of any soluble dehydrogenase enzyme, flavoprotein, cytochrome, or

cytochrome oxidase system by the substituted phenols has yet been found. The possibility that the substituted phenol anion might act like arsenate (Warburg and Christian, 1939) in substituting for phosphate in the triose phosphate dehydrogenase system was tested by Krahl (1947) with negative results. The current hypothesis is that the substituted phenols increase oxygen consumption by changing the relationship of one or more oxidative enzymes to the cell structure, in a way which gives a higher than normal overall rate of hydrogen (electron) transfer; the precise nature of this effect is not yet known (Krahl and Clowes, 1938b; Peiss and Field, 1948; Tepley, 1949). A foundation for further analysis of this oxidative stimulation has been laid by the observation that some stimulation of oxygen consumption by these agents can be obtained in cell-free granule systems from kidney (Loomis and Lipmann, 1948) and from Arbacia eggs (Crane and Keltch, 1949; Clowes, Keltch, Strittmatter, and Walters, 1949, 1950).

Efforts to account for the second phase of the substituted phenol effect, namely, the progressive inhibition of both oxygen consumption and cleavage in concentrations above the respiratory optimum, have revealed that these agents inhibit the following enzymatic activities: d-amino acid oxidase (Krahl, Keltch and Clowes, 1940), *Zwischen ferment* and cytochrome reductase (Haas, Harrer, and Hogness, 1942), phosphate accumulation and transfer by yeast cells (Hotchkiss, 1944; Spiegelman and Kamen, 1946). The concentrations required for inhibition in these systems were 10–100 times those required to inhibit cleavage of Arbacia eggs. Neither the cytochrome oxidase of Arbacia eggs (Krahl, Keltch, Neubeck, and Clowes, 1941) nor any water-soluble dehydrogenase is inhibited by physiologically active concentrations.

Observations which may be more pertinent to the supression of cleavage have just been reported by Clowes, Keltch, Strittmatter, and Walters (1949, 1950). They found that oxidative phosphorylation by a cell-free system from Arbacia eggs (see above) is inhibited by substituted phenols, in confirmation of the findings of Loomis and Lipmann (1948) upon kidney granules. The effects of the individual substituted phenols upon this cell-free phosphorylating system from the eggs are parallel to those of the same agents upon cleavage of the intact eggs in the following specific respects: (1) For each agent the block to both cleavage and phosphorylation is produced by concentrations just larger than that required for optimum oxygen consumption; (2) dinitrocarvacrol produces an increase in oxygen consumption in both the intact eggs and the particulate system, but blocks both cleavage and phosphorylation; on the other hand, its isomer, dinitrothymol, inhibits oxygen consumption in both systems and blocks both cleavage and phosphorylation; (3) p-nitrophenol stimulates oxygen consumption in both systems and blocks both cleavage and phosphorylation; its isomer, o-nitrophenol, is inactive toward oxygen consumption in both systems and blocks neither cleavage nor phosphorylation; (4) picric acid is completely inactive in both systems; (5) the concentrations of undissociated molecules required for each of five nitrophenols to block cleavage are exactly the same as those required to block phosphorylation.

That concentrations of 2,4-dinitrophenol ($5 \times 10^{-5}\ M$) which block phosphorylation in the cell-free system also block phosphorylation in the intact Arbacia egg was demonstrated by Abelson (1947) and by Villee, Lowens, Gordon, Leonard, and Rich (1949), who also found incorporation of P^{32} into all phosphate fractions to be inhibited; entrance of P^{32} into the DNA fraction being relatively most reduced.

Thus, there is reason to attribute the inhibition of cleavage to the interference with generation and transfer of "high energy" phosphate which the substituted phenols cause.

4. NAPHTHOQUINONES. The substance, 2-hydroxy-3(2-methyloctyl)-naphthoquinone-1,4 (SN 5949), prepared for test as an antimalarial, was found to have effects similar in some respects to those of the substituted phenols and in other respects to those of cyanide (Anfinsen, 1947). Below 7.4×10^{-8} *M*, respiration was stimulated; at 7.4×10^{-7} *M*, respiration of unfertilized and fertilized Arbacia eggs was inhibited by 50 per cent and 70 per cent, respectively; at the latter concentration cleavage was completely blocked.

Though the effects of this group of substances upon enzymes from eggs have not been studied, Ball, Anfinsen, and Cooper (1947) offer evidence that the naphthoquinones of this type may inhibit a respiratory step between cytochrome c and cytochrome b. It would be interesting to know whether echinochrome, the naphthoquinone pigment of the Arbacia egg, has similar effects.

5. IODOACETATE. Runnström (1935), using 0.03 *M* iodoacetate at pH values from 6.1 to 8.0, found that cleavage was abnormal and that the respiration of the fertilized eggs was strongly depressed; with maximal inhibition, the residual respiration is 40 per cent of the control rate. Addition of lactate or pyruvate plus the iodoacetate brings the respiration back nearly to normal, but the inhibition of cleavage is not overcome.

In sea water adjusted to pH 6.0 to permit penetration (see above), iodoacetic acid produces 50 per cent inhibition of cleavage at 1.7×10^{-3} *M* and 90 per cent inhibition at 3.4×10^{-3} *M*. At these concentrations, the rates of oxygen consumption are 67 and 54 per cent of the control values, respectively (Clowes and Krahl, 1940).

Ellis (1933) studied effects of iodoacetate and other reagents which combine with sulfhydryl groups upon cleavage of eggs of *Urechis caupo*.

Iodoacetate can combine with sulfhydryl groups and thereby inactivate enzymes which require this structure (Michaelis and Schubert, 1934; Hellerman, 1937; Barron and Singer, 1943). Its effects have not been studied on enzymes from the egg other than cytochrome oxidase, which it does not inhibit (Krahl, Keltch, Neubeck and Clowes, 1941).

6. URETHANE AND RELATED CARBAMATE DERIVATIVES. Interest in this group of substances was initiated by the observations of Warburg (1910), who showed that 0.0005 *M* phenylurethane could block cleavage of Strongylocentrotus while reducing oxygen consumption by 20 per cent (at most). The effects of a number of other carbamate derivatives upon cleavage and oxygen consumption have now been studied (Table VIII). Cleavage in Arbacia was blocked by phenylurethane when oxygen consumption was reduced to 70 per cent of normal, the critical concentration being 0.001 *M* (Clowes and Krahl, 1940). Determinations of effects of carbamates upon cleavage of Arbacia, made prior to 1932, are summarized by Harvey (1932).

Urethane is one of the narcotic agents which Fisher and his co-workers have used in an effort to show whether or not the oxidative activities associated with

cleavage (activity respiration) proceed via pathways different from the respiration of the unfertilized egg (resting respiration). They analyzed mathematically the relation between narcotic concentration, respiration, and cleavage and concluded that the activity respiration passes over a catalytic system which is either different from that operating in the resting egg or has a different affinity for the inhibitor. Observations consistent with the same conclusions were made upon chloral hydrate, azide, and sulfanilamide (Fisher and Henry, 1944; Fisher, Henry, and Low, 1944).

Cornman (1947) determined effects of various carbamates on cleavage of Lytechinus.

The enzymatic mechanism underlying the specific effect of the carbamates on cleavage remains to be defined, but is obviously worthy of study.

7. Dyes. After the demonstration by Harrop and Barron (1928) that dyes capable of oxidation-reduction could enhance oxygen consumption of red cells, the effects of dyes upon Arbacia eggs were measured from a number of points of view: to determine whether dyes could substitute for oxygen as hydrogen acceptors in presence of respiratory inhibitors (Brooks, 1941); to determine whether the excess oxygen uptake could be used to accelerate cleavage and development (Clowes and Krahl, 1936; Brooks, 1941; Shapiro, 1948a); and as an aid in specifying the mechanism by which oxygen transfer is carried out in the eggs (Barron, 1929, 1932; Runnström, 1935; Korr, 1937; Brooks, 1941).

The individual investigators agree that, in concentrations from 10^{-4}–10^{-3} *M* at pH 7–8, methylene blue, pyocyanine, and a number of other dyes can increase oxygen consumption of both unfertilized and fertilized Arbacia eggs, but they do not agree completely in regard to the effects of the same agents upon cleavage and developmental processes. Clowes and Krahl (1936), studying cleavage over the three hour period after fertilization, found all dyes tested to produce irreversible cleavage block at the optimum concentration for respiratory stimulation. Shapiro (1948a), studying time required to reach first cleavage, likewise found an inhibition which, however, was reversible. The difference between the results of Shapiro and those of Clowes and Krahl may well lie in the length of time the eggs were exposed to the reagent. Brooks (1941, 1943), studying effects of KCN, CO, and methylene blue upon unfertilized eggs, and the early cleavage, morula, blastula, gastrula, and pluteus stages, found 0.0001 *M* methylene blue to increase the rate of development during early cleavage stages and to increase the size of the pluteus of Arbacia. Shapiro (1948a) could not confirm this favorable effect upon cleavage.

Brooks (1947) reported artificial parthenogenesis of eggs of *Urechis caupo* and *Strongylocentrotus purpuratus* by oxidation-reduction dyes.

8. Barbiturates and Local Anesthetics. The effects of 30 barbituric acid derivatives (Clowes, Keltch, and Krahl, 1940) and of 16 local anesthetics (Krahl, Keltch, and Clowes, 1940a) upon cleavage of the Arbacia egg were determined in the course of a study of their mechanism of penetration into living cells. The results are given in Tables VI and VII.

With a representative barbiturate, 5-isoamyl-5-ethyl barbituric acid, the oxygen consumption was 75 per cent of control when division was inhibited 50 per cent and 20 per cent of control when division was inhibited completely. A typical local

anesthetic, γ-(2-methylpiperidyl)-propyl benzoate, produced 64 per cent inhibition of cleavage while oxygen consumption remained at, or slightly above, normal. Thus, the local anesthetic bases, like azide, sulfide, and the carbamates, have specific inhibitory effects upon cleavage which are not dependent upon inhibition of oxygen consumption.

The actions of barbiturates and local anesthetics upon enzyme systems of the Arbacia egg have not been studied.

TABLE VI

Concentrations of substituted barbituric acids required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at 20° C. and approximately pH 7.8–8.0 [1]

(From Clowes, Keltch, and Krahl, 1940)

Substituted barbituric acid	pK′ at 20° for sea water	Final pH of medium	Total concentration for 50 per cent inhibition of cleavage
			moles per l. × 10^4
1. 5-ethyl-5-(β-ethylhexyl)	7.75	8.00	2
2. 5-allyl-5-(α-methylbutyl)	7.92	7.81	2
3. 5-benzyl-5-isopropyl	7.82	7.97	4
4. 5-(β-bromoallyl)-5-(α-ethylpropyl)	7.58	8.00	5
5. 5-ethyl-5-(α-methylbutenyl-2)	7.78	7.99	5
6. 5-(β-methylallyl)-5-(α-methylbutyl)	7.80	7.86	7
7. 5-ethyl-5-hexyl	7.66	8.00	8
8. 5-ethyl-5-(α-methylbutyl)	8.02	8.00	12
9. 5-(β-bromoallyl)-5-(α-methylpropyl)	7.60	8.00	13
10. 5-ethyl-5-(α-ethylpropyl)	7.89	7.98	14
11. 5-allyl-5-benzyl	7.06	7.92	15
12. 5-amyl-5-ethyl	7.82	7.93	15
13. 5-(α, γ-dimethylbutyl)-5-ethyl	7.99	7.90	15
14. 5-isoamyl-5-ethyl	7.78	8.04	15
15. 5-cyclopentenyl-5-ethyl	7.98	7.97	17
16. 5-ethyl-5-(β-phenylethyl)	7.74	7.99	22
17. 5-allyl-5-isobutyl	7.54	8.00	39
18. 5-isobutyl-5-(β-methylallyl)	7.62	7.93	40
19. 5-butyl-5-ethyl	7.76	7.97	40
20. 5-sec-butyl-5-crotyl [2]	—	8.03	42
21. 5-(β-bromoallyl)-5-isopropyl	7.59	8.00	48
22. 5-ethyl-5-cyclohexenyl	7.36	8.00	58
23. 5-ethyl-5-phenyl	7.26	7.92	95
24. 5-allyl-5-isopropyl	7.73	7.97	98
25. 5-cyclohexenyl-1,5-dimethyl	8.24	8.74	104
26. 5,5-diallyl	7.62	7.96	160
27. 5-ethyl-5-isopropyl	7.90	8.02	162
28. 5-ethyl-5-piperidyl	7.56	7.92	200
29. 5,5-diethyl	7.75	7.94	320

[1] The concentrations required at pH 6.4–6.7 and at pH 8.6–8.9 were also determined.

[2] Derivative of thiobarbituric acid rather than of barbituric acid itself.

9. MISCELLANEOUS. The effects of many other agents, with unknown effects upon metabolic enzymes, have been tested upon respiration and cleavage of Arbacia eggs.

Since these do not contribute directly to the principal objective of this paper, namely, to elucidate the metabolic processes by which energy is provided by cleavage, they will not be dealt with here in detail. But all the miscellaneous agents which have been employed in the period 1932–1949 are listed below, and quantitative data for them, where available, are given in Tables VIII and IX.

TABLE VII

Concentrations of local anesthetics required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at 20° C. and approximately pH 7.8–8.0[1]

(From Krahl, Keltch, and Clowes, 1940)

Local anesthetic	pK′ at 20° for sea water	Final pH of medium	Total concentration for 50 per cent inhibition of cleavage
			moles per l. × 10^4
1. N,N-Diethyl-N′-(2-butyloxy-quinoline-4-carboxyl)-ethylenediamine ('Dibucaine')	5.03	7.99	0.06
2. α-Piperidyl-β,γ-dicarbanilido-propanediol ('Diothane')	5.84	7.94	0.3
3. β-Dimethylaminoethyl-p-butylaminobenzoate ('Tetracaine')	5.50	7.98	0.9
4. 4,4′-Diethoxy-N,N′-diphenyl-amidine ('Phenacaine')	4.78	7.90	1.3
5. 2-Benzoxy-2-methyl-1-dimethylaminobutane ('Stovaine')	5.42	7.90	1.3
6. γ-Dibutylaminopropyl-p-aminobenzoate ('Butacaine')	4.90	7.91	3.1
7. 4-Benzoxy-2,2,6-trimethylpiperidine ('β-Eucaine')	4.27	7.90	4.5
8. γ-Diethylaminopropylcinnamate ('Apothesine')	4.06	7.93	5.2
9. β-Diethylaminoisohexyl-p-aminobenzoate ('Panthesine')	4.44	7.90	7.0
10. 2-Benzoxy-2-dimethylaminomethyl-1-dimethylaminobutane ('Alypin')	4.55	7.92	7.2
11. N-Methyl-l-benzoylecgonine ('Cocaine')	4.97	7.78	10
12. β,β-Dimethyl-γ-diethylamino-propylbenzoate ('Larocaine')	4.21	7.91	10
13. γ-(2-Methylpiperidyl)-propylbenzoate ('Metycaine')	4.18	8.00	13
14. Ethyl-p-aminobenzoate ('Benzocaine')	10.82	7.97	15
15. β-Diethylaminoethyl-p-aminobenzoate ('Procaine')	4.73	7.93	23
16. 2-(p-Aminobenzoxy)-2-methyl-1-dimethylaminobutane ('Tutocaine')	4.82	7.93	45

[1] The concentrations required at pH 6.4–6.7 and at pH 8.6–8.9 were also determined.

Acetyl salicylic acid—Budington (1935)

Acids and bases—Clowes, Keltch, and Wade (1933, 1934); Keltch, Wade, and Clowes (1933)

Amyl alcohol—Clowes and Keltch (1941)

Caffeine and related compounds—Cheney (1945, 1948)

TABLE VIII

Effect of various substances upon oxygen consumption and cleavage of fertilized Arbacia eggs

Agent	T° C.	pH	Oxygen consumption			Cleavage		
			Maximal change produced	Conc. required to produce maximal change	Change at conc. required to inhibit cleavage maximally	Maximal inhibition	Conc. required to produce maximal inhibition	Reversibility of maximum inhibition
			per cent of control	moles per 1. × 10^4	per cent of control	per cent of control	moles per 1. × 10^4	
Miscellaneous								
n-Amylalcohol [1]	20	8.0				100	660	none
Caffeine [2]	25	7.9	−60	250	−60	100	250	not stated
Carbon dioxide [3]	21		−80		−70	—	—	not stated
Chloral hydrate [6]	24	7.3–8.3	−80	1000	−60	100	63	partial
Chloroform [1]	20	8.0				100	160	none
Colchicine [4]						100	1	not stated
Colchicine [5]						100	2	not stated
Ethyl carbamate [6]	24	7.3–8.3	−75	6300	−30	100	1400	yes
Hydroxyethylapocupreine [1]	20	7.8				100	41	none
p-(α-Methylbutylamino) benzoate [1]	20	7.8				100	10	partial
Penicillin [7] (non-crystalline)	20	7.5				100	5000 [7]	not stated
Phenylmercury nitrate [1]	20	6.9–8.6				100	0.02	none
Podophyllotoxin [8]						100	0.01	not stated
Tetrachlorohydroquinone [1]	20	7.9				100	0.6	none
Tetrachloroquinone [1]	20	7.9				100	1	none
Usnic acid [9]						100	3	not stated
Sulfonamides and related compounds								
n-Butylsulfone-p-phenetidide [1]	20	7.9				65	20	yes
Diaminodiphenylmethane [1]	20	7.9				100	50	yes
Diaminodiphenylsulfone [1]	20	7.9	−43	20	−35	60	9	yes
Ethylsulfone-p-phenetidide [1]	20	7.9				100	16	yes
Methylsulfone-p-phenetidide [1]	20	7.9				40	41	yes
p-Nitrosobenzenesulfonamide [1]	20	7.9			0	100	2	none
n-Propylsulfone-p-toluidide	20	7.9				100	20	yes
Sulfanilamide [10]	25	8.0	−48	400	−45	100	400	yes
Sulfanilylethanolamine [1]	20	7.9				60	280	none
N^4-Trichloroacetyl-sulfanilamide [1]	20	7.9				70	20	none

TABLE VIII—*Continued*

Agent	T° C.	pH	Oxygen consumption			Cleavage		
			Maximal change produced	Conc. required to produce maximal change	Change at conc. required to inhibit cleavage maximally	Maximal inhibition	Conc. required to produce maximal inhibition	Reversibility of maximum inhibition
			per cent of control	moles per l. × 10^4	per cent of control	per cent of control	moles per l. × 10^4	
Dyes								
o-Cresol indophenol [12]	20	7.9	+75	3	+30	100	1	none
Dimethyl-p-phenylene diamine [12]	20	7.9	+125	10	+120	100	2	none
Methylene blue [12]	20	7.9	+60	10	+50	100	5	none
Neutral red [12]	20	7.9	+25	1	0	90	10	none
Pyocyanine [12]	20	7.9	+200	5	+90	100	30	none
Pyocyanine [11]	—	—	+96	5	—			
Tetramethyl-p-phenylene diamine [12]	20	7.9	+250	10	+250	100	10	none
Dithiocarbamates								
sec-Butyl ethyldithiocarbamate [1]	20	7.9				80[13]	0.3	none
Di-n-butyldithiocarbamate [1]	20	7.9				100	41	none
Diethyldithiocarbamate [1]	20	7.9	+40	1	0	85[13]	0.3	none
Di-isoamyldithiocarbamate [1]	20	7.9				100	5	none
Dimethyldithiocarbamate [1]	20	7.9				50	81	none
Di-n-propyldithiocarbamate [1]	20	7.9				100	0.3	none
N,N′-Dimethyl-(2,4-dinitrophenyl)-dithiocarbamate [1]	20	7.9				100	20	none
Isoamylethyldithiocarbamate [1]	20	7.9				100[13]	0.2	none
Isobutylethyldithiocarbamate [1]	20	7.9				100[13]	0.3	none

[1] Clowes and Keltch (1939, 1940, 1941). [2] Cheney (1945, 1948).

[3] Root (1930). The maximal effect on oxygen consumption was measured at 180 mm. CO_2, 0.0018 *M* $NaHCO_3$, pH 5.50. The maximal effect on cleavage as measured by the cleavage delay method was obtained at 40 mm. CO_2, 0.0018 *M* $NaHCO_3$, pH 6.15, at which point oxygen consumption was reduced by 70 per cent.

[4] Nebel (1937). [5] Beams and Evans (1939). [6] Fisher and Henry (1944).

[7] Henry and Henry (1945). The concentration is stated in Oxford units per ml.

[8] Cornman (1947). [9] Marshak and Harting (1948). [10] Fisher, Henry, Low (1944).

[11] Runnström (1935b). Pyocyanine at 5 × 10^{-4} *M* also increased oxygen consumption of unfertilized eggs by 210 per cent.

[12] Clowes and Krahl (1936).

[13] A smaller degree of inhibition was produced by higher concentrations of this substance. This effect, while reproducible, is not understood.

TABLE IX

Delay in first cleavage of fertilized Arbacia eggs produced by various chemical agents

Agent	Concentration	T° C.	pH	Delay in first cleavage
				minutes
CO_2-bicarbonate [1]	10 mm. CO_2-0.0018 *M* $NaHCO_3$	20	6.76	16
	40 mm. CO_2-0.0073 *M* $NaHCO_3$		6.70	44
	80 mm. CO_2-0.0143 *M* $NaHCO_3$		6.67	108
	20 mm. CO_2-0.0018 *M* $NaHCO_3$		6.41	30
	80 mm. CO_2-0.0073 *M* $NaHCO_3$		6.41	260
Methylene blue [2]	1×10^{-4} *M*	26		2
	2×10^{-4} *M*			5
	4×10^{-4} *M*			10
Toluylene blue [2]	4×10^{-4} *M*	26		57
Ascorbic acid [3]	4×10^{-5} *M*	26.1		6
	2×10^{-4} *M*			7
	9×10^{-4} *M*			16
	2×10^{-3} *M*			30

[1] Haywood and Root (1932).
[2] Shapiro (1948a).
[3] Shapiro (1948b).

Carbon dioxide—Smith and Clowes (1924); Root (1930); Haywood and Root (1930, 1932)

Carcinogenic hydrocarbons—Keltch, Krahl, and Clowes (1937)

Chloral hydrate—Fisher and Henry (1944)

Chloroform—Clowes and Keltch (1941)

Colchicine—This substance is said to stop mitosis at metaphase, Nebel (1937); Beams and Evans (1940)

Dithiocarbamates—Clowes and Keltch (1941)

Ethyl alcohol—Waterman (1936)

Hydroxyethylapocupreine—Clowes and Keltch (1940)

Hypotonic sea water—Cornman (1943)

Leukotaxine—Menkin (1940)

Malonic acid—Rulon (1948)

Malononitrile—This substance interferes with phosphate transfer in intact eggs, Villee, Lowens, Gordon, Leonard and Rich (1949)

Metal ions—Alkali metals (Li, Na, K)—Chambers and Chambers (1938); Runnström (1935c); Moore, Bliss, and Anderson (1945); *copper*—Glaser (1924); Finkel, Allee, and Garner (1942); Runnström (1939); *other heavy metals*—Page (1929); Waterman (1937)

Nitrogen mustards—Agents of this group have been studied by Barron, Seegmiller, Mendes, and Narahara (1948) and by Hutchens and Podolsky (1948). No inhibition of oxygen consumption of the eggs was observed. Hutchens and Podolsky found the cleavage over a 3 hour period to be reduced to 75 per cent of control

by tris-(β-chloroethyl)amine at 3×10^{-5} *M* and by various bis-(β-chloroethyl) amines at 2×10^{-4} *M*.

Penicillins—Non-crystalline samples of penicillin had a slight inhibitory effect on cleavage at concentrations several thousand times those required to stop growth of sensitive bacteria (Henry and Henry, 1945), but highly purified samples of various penicillins were virtually without effect upon either respiration or cleavage (Clowes and Keltch, 1946).

Phenylmercury nitrate—Clowes and Keltch (1940)

Podophyllotoxin—Cornman (1947)

Pressure—Marsland (1939)

Rhodamine B—Clark (1940)

Sulfhydryl-blocking agents—Barron, Nelson, and Ardao (1948); Clowes and Keltch (1940)

Sulfonamides—Fisher, Henry, and Low (1944); Clowes and Keltch (1939)

Temperature—Rubenstein and Gerard (1933); Hoadley and Brill (1937); Tyler and Humason (1937); Borei (1949); Borei and Lybing (1949)

Tetrachlorohydroquinone—Clowes and Keltch (1940)

Ultraviolet light—Giese (1938a, 1938b, 1939); Harvey and Hollaender (1938)

Uranyl nitrate—Effects of this substance upon egg respiration have not been reported, but it inhibits respiration of Arbacia sperm (Benedict and Barron, 1946). It also inhibits phosphate transfer in the intact egg (Villee, Lowens, Gordon, Leonard, and Rich, 1949).

Usnic acid—This substance inhibits cleavage and phosphate transfer (Marshak and Harting, 1948), and also desoxyribonuclease (Marshak, 1949).

Vitamin C—Shapiro (1948b)

X-rays—Henshaw (1932, 1938, 1939); Henshaw and Francis (1936); Cohen (1939); Evans (1940); Evans and Beams (1939)

The following agents have been found to be without effect upon cleavage of fertilized Arbacia eggs when tested at the molar concentrations shown, which were the highest concentrations employed (Clowes and Keltch, 1939–1941): diethylmethylcarbinylurea, 8×10^{-3}; 4,4′-dinitrobenzophenone, 6×10^{-4}; 2,4-dinitrophenylmercaptobenzothiazole, 2×10^{-3}; morphine, 2×10^{-2}; phenacetine, 8×10^{-3}; porphyrexide, 1×10^{-3}; 2-sulfanilylaminothiazole, 5×10^{-4}; N-sulfanilylbenzamide, 5×10^{-4}; sulfapyridine, 8×10^{-3}; synthalin, 2×10^{-3}.

G. Summary

1. This review deals with the general question of relation of metabolic activities to cleavage in the Arbacia egg. According to current biochemical concepts the answer to this question would be found in complete information about the following subjects:

a. The identity and interrelationships of the catalysts by which the eggs derive energy from foodstuffs.

b. The reactions by which energy is transferred to make possible the synthesis of structural and functional elements of the egg, such as carbohydrates, proteins, lipids, coenzymes, nucleic acids and other substances, many of which may yet be unknown.

c. The chemical and physical chemical rearrangements of these structural functional elements which lead to the biological event of cleavage.

The third subject is completely beyond investigation by present techniques, and the second is just being opened to investigation in the living egg by application of isotopic labelling techniques. This review is concerned, therefore, chiefly with the nature and interrelationships of the catalysts by which Arbacia eggs derive energy from foodstuffs.

2. The metabolic catalysts which have been shown to be *available* in the Arbacia egg for deriving energy from foodstuffs are:

a. An enzyme with some, but not all, of the properties of the cytochrome oxidase of other animal tissues. After extraction from the egg it oxidizes cytochrome c, but whether this activity is related to its biological function in the egg is an open question, as no biochemically significant quantity of cytochrome c has yet been conclusively demonstrated in the eggs, although it is present in the sperm.

The enzyme is poisoned by carbon monoxide, cyanide, azide, and sulfide, but not by copper-binding reagents. Its relative affinities for oxygen and carbon monoxide are, in the cell-free state, the same as those for cytochrome oxidase of mammalian muscle but, in the intact Arbacia egg, these relative affinities are intermediate between those of the usual cytochrome oxidase and those of other iron-porphyrin compounds such as hemoglobin.

b. The coenzymes, flavin-adenine dinucleotide, diphosphopyridine nucleotide, and diphosphothiamine. The properties of the enzymes of which these coenzymes are parts have not been investigated.

c. A mechanism for oxidative disposal of pyruvate.

d. An enzyme complex which can oxidize oxalacetate, α-ketoglutarate, or succinate with concomitant disappearance of inorganic phosphate, presumably via generation of "high energy" phosphate bonds and esterification of suitable phosphate acceptors.

e. The following enzymes which have undefined roles with respect to energy production and to chemical syntheses in the egg: dehydrogenases for various substrates other than those already mentioned; phosphatases; ribonuclease; desoxyribonuclease; catalase; peptidases.

The distribution of various enzymes in the egg has been studied with very suggestive findings. In the unfertilized or recently fertilized egg the total reducing activity, cytochrome oxidase, and desoxyribonuclease are quantitatively most abundant in the cytoplasmic matrix, i.e. in solution or upon the small granules less than 1 μ in diameter. As development proceeds, a progressively greater fraction of the enzymes are associated with larger granules of the type which stain as mitochondria. In view of these observations and the fact that the capacity for oxidative phosphorylation is found, in the adult mammalian tissues so far studied, to be associated with mitochondrial particles, the suggestion arises that localization of enzymes on specific particles may parallel development of specific function.

3. What is the *actual* pathway connecting enzyme activities with cleavage? Such answers to this question as are available come largely from experiments designed to block a specific metabolic enzyme system and to observe the degree to

which the egg can or cannot cleave when deprived of this enzyme or group of enzymes.

A note of caution should be emphasized here. The problem of explaining physiological effects in chemical terms is an exceedingly difficult one. It involves the demonstration not only that a given effect of a reagent upon an enzyme is *sufficient* to explain the physiological effect, but also the demonstration that the chemical effect offered as explanation is the only one possible, i.e. is a *necessary* explanation. Only the sufficiency criterion is usually met. In view of these considerations it is clear that conclusions about the relation of enzymes to function, which are based on action of inhibitors, must be only provisional. With this reservation in mind, the main findings about enzymatic activities and cleavage may be summarized.

a. For energy to support cleavage, eggs of Arbacia are completely dependent on aerobic processes. Like other animal cells, the eggs can form lactic acid but this process cannot support cleavage.

The nature of the foodstuffs oxidized is not settled; evidence for oxidation of carbohydrate or its phosphorylated breakdown products, of fats, or of amino acids has been offered, but is not conclusive.

b. Evidence for participation of two enzyme systems in oxidative energy-yielding reactions has been presented.

(1) *The cytochrome oxidase system.* The principal indication for participation of this oxidative step is that both the inhibition of oxygen consumption and the inhibition of cleavage by carbon monoxide are *light reversible*. Cytochrome oxidase is the only metabolic catalyst known to be subject to light-reversible inhibition by carbon monoxide.

(2) *An oxidative phosphorylation system.* The principal evidence is the highly specific parallel between inhibition of cleavage and inhibition of a cell-free phosphorylating system from the eggs by a series of substituted phenols. Supporting evidence is found in the fact that these agents likewise block phosphorylation and phosphate transfer in the intact fertilized egg.

c. In an effort to reveal the metabolic steps essential for cleavage, comparisons of the metabolic activities of unfertilized and fertilized eggs have been made.

The oxygen consumption of intact Arbacia eggs increases upon fertilization to a degree which depends on the temperature, by a factor of about 10 times at 11° C., by a factor of about 2 times at 30° C., and negligibly at 32° C. The higher respiration of the fertilized egg is relatively more sensitive than that of the unfertilized both to inhibitors of cytochrome oxidase, such as cyanide and carbon monoxide, and to inhibitors which act at other loci, such as narcotics.

The concentration of cytochrome oxidase is the same in unfertilized and fertilized eggs, and in great excess relative to oxygen consumption of intact eggs. The concentration of total dehydrogenase activity, as measured by ability to reduce ferricyanide, is likewise unchanged upon fertilization.

The ability to dispose of pyruvate, on the other hand, appears to be increased following fertilization.

From these and related facts, all observers appear to agree that the increase in oxygen consumption upon fertilization is the consequence of the introduction of a new or accelerated step between some substrate, perhaps pyruvate, and cytochrome

oxidase. The nature of the new or accelerated step, a frequent subject of speculation, is unknown. It is not known to what extent, if any, the energy liberated by the excess oxygen consumption is used for synthetic processes and for cleavage.

The precise mechanism by which oxidative energy is made available for cleavage remains to be defined.

4. In the interest of completeness, and to provide a foundation for the further study of relation of metabolic activities and cleavage, the effects of all agents so far tested upon oxygen consumption and cleavage of Arbacia eggs have been recorded, even though their effects upon specific enzymatic activities frequently still await investigation. Examples of the viewpoints from which these substances have been investigated, and of the agents employed, are:

a. To demonstrate participation of various enzyme systems in yielding energy for cleavage: poisons for cytochrome oxidase such as cyanide, carbon monoxide, azide, sulfide; poisons for phosphorylation processes such as substituted phenols, azide, usnic acid; and poisons for other metabolic enzymes such as naphthoquinones and iodoacetate.

b. To learn more about the mechanisms by which physiologically active substances affect growth and cell division: acids and bases, carbamates, colchicine, nitrogen mustards, sulfonamides, penicillin.

c. To determine the form in which toxic ionizable substances penetrate living cells such as Arbacia eggs: substituted phenols, barbiturates, local anesthetics, other weak acids and bases. These agents appear to gain access to the interior of the Arbacia egg chiefly, if not entirely, as undissociated molecules.

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STUDIES ON THE PHYSIOLOGY OF UCA RED CHROMATOPHORES[1]

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Red chromatophores are present in considerable abundance in most individuals of *Uca pugilator* that are collected in the salt marshes in and near Woods Hole, Massachusetts. The abundance is sufficient to provide the animals with a distinctly reddish tint. Almost nothing has been reported concerning the responses of these chromatophores to background or to other factors, nor have any systematic studies been made as to the nature of the control of these chromatophores within the organism. Carlson (1936) reported that the red pigment was concentrated in eyestalkless *Uca pugilator*. This was confirmed by Abramowitz (1937) who reported further that injection of eyestalk extract into eyestalkless specimens caused red dispersion.

In view of the rapidly increasing information on the physiology of the black and white chromatophores of the fiddler crabs, and the various sources of chromatophorotropins involved, it seemed wise at this time to examine the red chromatophores, comparing them with the other chromatophores in their responses, to learn to what extent they were governed by the same or similar mechanisms, and to what extent by quite different ones.

Responses to Black and White Backgrounds

A number of experiments were performed to determine the character and magnitude of changes in the state of red chromatophores in response to black and white backgrounds. In a typical experiment, twenty fiddler crabs were divided into two lots of ten each; one lot was placed in a black enamelled container, the second in a white one. After about three hours in these situations the average state of dispersion of the pigment of the red chromatophores was recorded, using the well-known chromatophore index in which a chromatophore with its pigment in punctate condition is designated as 1, and one with its pigment completely dispersed, as 5, with 2, 3, and 4 describing three intermediate states. The backgrounds of the two lots of animals were then interchanged and the average state of the red pigment in the two lots determined after one, two, and three hours. The results of this experiment, conducted at an illumination of 100 f.c., are shown in Figure 1. It is readily seen that the pigment responds extensively, in fact through almost its entire normal range of change, during the daytime, at constant incident illumination, and in response simply to these changes in albedo.

In the foregoing characteristic, the red chromatophores of *Uca pugilator* differ quantitatively in their responses from the black and white chromatophores, whose responses to albedo, when incident illumination, temperature, and time in the daily

[1] This investigation was supported by a research grant from the graduate school of Northwestern University.

rhythmical cycle of color change are constant, are of relatively small magnitude (Brown and Sandeen, 1948).

The red chromatophores of *Uca*, therefore, appear in their responses well adapted for alteration of the shade of the body to accord more closely with that of the background upon which the animal is located.

Diurnal Rhythm of Red Chromatophore State

In conditions of constant darkness, the red chromatophores of *Uca pugilator* exhibit a very striking persistent rhythmicity of activity. This rhythm is illustrated in Figure 2A (solid line) in which it is readily observed that the pigment is more or less broadly dispersed by day and almost completely concentrated by night. The darkened blocks immediately above and below the plotted rhythm indicate the hours 6 P.M. to 6 A.M. The bottom band in the figure indicates the experimental illumination which, in this instance, is constant darkness. The amplitude of the rhythm was, in fact, substantially greater in this case than that of the rhythm simultaneously occurring in the black chromatophores of these animals (broken line in Figure 2A). The two rhythms are, however, clearly seen to be in phase with one another.

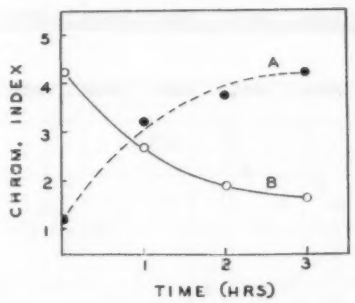

Figure 1. Changes in the red chromatophores in response to change of background: A. White to black; B. Black to white.

The rhythm of the red chromatophores was observed to persist with no loss in amplitude, and completely synchronized with the solar day-night cycle for four days.

In an attempt to demonstrate whether the red and black chromatophores were able, in a normal animal, to show activities in opposite directions to one another at any given moment, ten animals were exposed to twelve hours of illumination by night and to twelve hours of darkness by day over a period of three days. They were maintained during the experiment upon a white enamelled background. The results of this experiment are shown in Figure 2B between points *a* and *b*. The red chromatophores (solid line) showed dispersion of their pigment during the darkened day-time period, and concentrated their pigment during the illuminated night-time period in response to the white background.

The black chromatophores (broken line), on the other hand, governed predominantly by the endogenous persistent rhythmicity, altered the phase of their

rhythm in response to the reversed condition of periods of illumination in such a manner as to show their maximally concentrated phase at mid-day rather than at mid-night (Brown and Webb, 1948, 1949), and hence the rhythms of activity of the two pigments are seen to be out of phase with one another as long as the reversed periods of illumination are continued.

It is also seen from Figure 2B, beginning at point *b*, that when the animals are returned to constant darkness after the three complete cycles of reversed illumina-

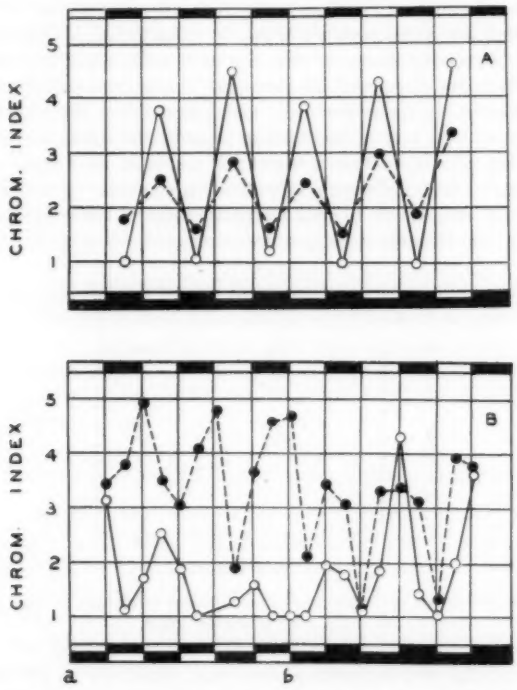

FIGURE 2. A. The normal persistent rhythmicity of red (solid line) and black (broken line) chromatophores in constant darkness. See text for explanation of diagrams.

B. Behavior of red (solid line) and black (broken line) chromatophores in response to a series of periods of reversed illumination beginning at *a*, followed by constant darkness beginning at *b*.

tion, the black pigment, as a result of a shift in the phase of the endogenous source of the rhythm, continues to exhibit a persistent 24-hour cycle of activity with maximum concentration of the pigment now at about 7 A.M. Under these conditions the red pigment also undergoes a similar rhythmical activity completely in phase now with the black. This rhythm was observed to persist unchanged for more than a week.

These results clearly indicate that the red pigment, other environmental stimuli removed, is under a very effective control by an endogenous mechanism of persistent

rhythmicity, and that in the absence of such other stimuli the various phases of red chromatophore activity are controlled and experimentally shifted in a manner quite parallel to that found for the black chromatophores.

It is also evident from these observations that the red pigment is capable of exhibiting an activity independent of that of the black; indicating, in addition, some difference in the factors contributing to their control.

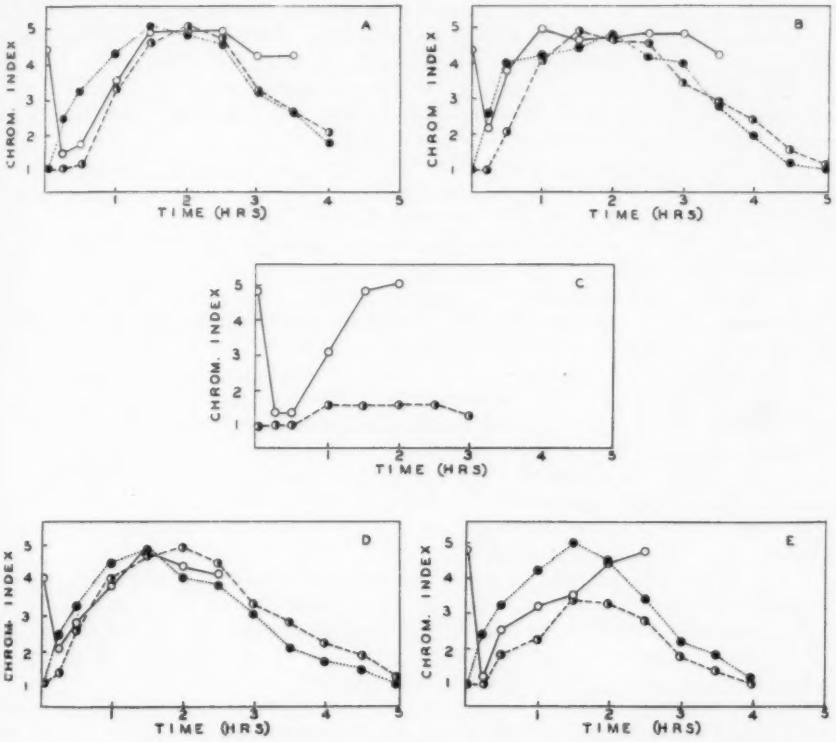

FIGURE 3. Responses of chromatophores of eyestalkless (red chromatophores—dashed line; black chromatophores—dotted line) and black-adapted intact *Uca* (red chromatophores—solid line) to injection of extracts of A. sinus glands, B. optic ganglia, C. circumoesophageal connectives, D. brain, and E. thoracic cord.

SOURCES OF RED-PIGMENT-INFLUENCING FACTORS

The sinus glands and various portions of the central nervous system appeared to be the most likely sources of chromatophorotropins influencing red pigment, in view of their demonstrated activities on white and black pigments in both this species (Brown, 1948) and another crab (Bowman, 1949). Consequently, a series of experiments was designed to determine what influences, if any, extracts of these organs might show.

Sinus glands, optic ganglia from which sinus glands had been carefully removed, brains, connectives, and thoracic cords were removed from a number of specimens and triturated in sea water in such volumes that a single dose (0.05 ml.) to be injected later into an assay animal would contain the equivalent of one-third of the complement of that tissue taken from a single animal. Such an extract was referred to as possessing a concentration of 1/3. The various extracts were then each assayed on (1) five *Uca pugilator* whose eyestalks had been removed 24 hours previously and whose red chromatophores were in an initial state of 1, and (2) five normal animals which were adapted to a black background and which were maintained upon a black background throughout the assay.

The states of the red chromatophores were determined at the time of the injection and then at 15 minutes, 30 minutes, and at 30-minute intervals thereafter until the chromatophores returned to their initial states.

The results of these experiments are summarized in Figure 3. The solid lines represent the averages for the black-adapted assay animals whose red pigment was initially dispersed, and the dashed lines represent the averages for the eyestalkless ones whose pigment was initially concentrated. The dotted lines show the behavior of the black chromatophores in the eyestalkless assay specimens.

It is readily seen that all of the extracts exhibited a substantial influence on the red chromatophores, an effect not shown either by sea-water injections or injections of extracts of muscle tissue which were used as controls.

The influence of all the active extracts showed two characteristics in common. The first was that they all exerted a rather strong concentrating influence upon the red pigment that was initially dispersed in the black-adapted assay animals. Undoubtedly the same type of activity accounted for the significant delay in the initiation of dispersion of red pigment in the eyestalkless assay animals whose red pigment was initially strongly concentrated. There is, in this latter connection, a rather close correlation between the magnitude of the red-pigment-concentrating action and the extent of this initial delay.

The second type of activity, one which was most conspicuously evident in the eyestalkless assay animals, was that of strong dispersion of red pigment. This action was also evident in the black-adapted animals in the form of the dispersion of the red pigment to a condition greater than that of the initial and final states, following the initial period of concentration.

Closer inspection of Figure 3 shows that the five extracts did not exhibit equal red-pigment-concentrating actions on the black-adapted assay animals. It can be seen that the apparent order of decreasing activity in this regard was as follows:

Thoracic cord > Connectives > Sinus glands > Brain > Optic ganglia

The foregoing order of red-concentrating activity was not the same, or even the simple inverse, of the order of red-pigment-dispersing activity which appears to be:

Optic ganglia > Brain > Sinus glands > Thoracic cord > Connectives

It would appear from these data that all of the portions of the nervous system, and the sinus glands as well, possess two different principles which are responsible for these two observed influences on red pigment.

Additional support for the dual activity of brain and thoracic cord extracts upon red pigment is seen in Figure 4, A and B, in which extracts of these organs were

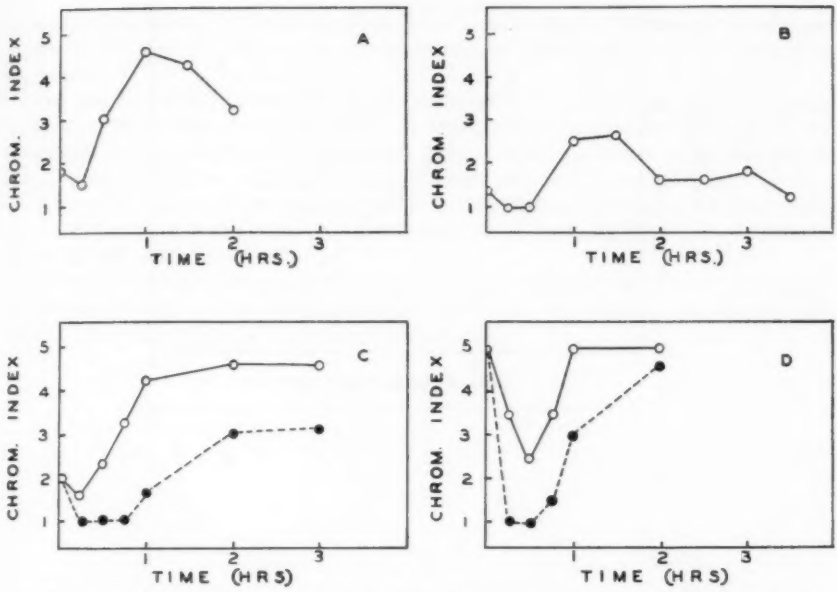

FIGURE 4. Responses of intact white-adapted *Uca* to extracts of A brain and B thoracic cord. C. Responses of the red chromatophores of eyestalkless *Uca* to alcohol-soluble (broken line) and alcohol-insoluble (solid line) fractions of sinus glands. D. Same as C but now responses of intact black-adapted *Uca*.

injected into normal white-adapted assay animals maintained upon a white background. Again, the same duality of activity of the extracts is observed.

A partial separation of these two principles appears to have been effected by the preparation of two types of extracts of sinus glands: one an ethyl-alcohol-soluble fraction and the other an ethyl-alcohol-insoluble fraction. Figure 4, C and D, shows the results of injecting these two fractions in equivalent concentrations into (C) eyestalkless assay animals selected to show slight red dispersal, and (D) black-adapted assay animals. In both instances the alcohol-soluble fraction appeared to possess a distinctly larger proportion of the red-pigment-concentrating activity, and the alcohol-insoluble fraction to possess the more black-dispersing action.

In an attempt to compare quantitatively the various portions of the nervous system and the sinus glands, one with another, with respect to their red-dispersing activity, extracts of sinus glands and of optic ganglia with sinus glands carefully removed were made up in concentrations of 1/3 as in the earlier experiments. A series of dilutions of these extracts was made to yield concentrations of 1/3, 1/9, 1/27, 1/81, 1/243, and 1/729. Each concentration of each organ was injected into five eyestalkless assay animals. This experiment was repeated three times. In Figures 5, A and B, are shown the relationships between the log concentration of the extract and the red-pigment-dispersing activity. The relationship is seen

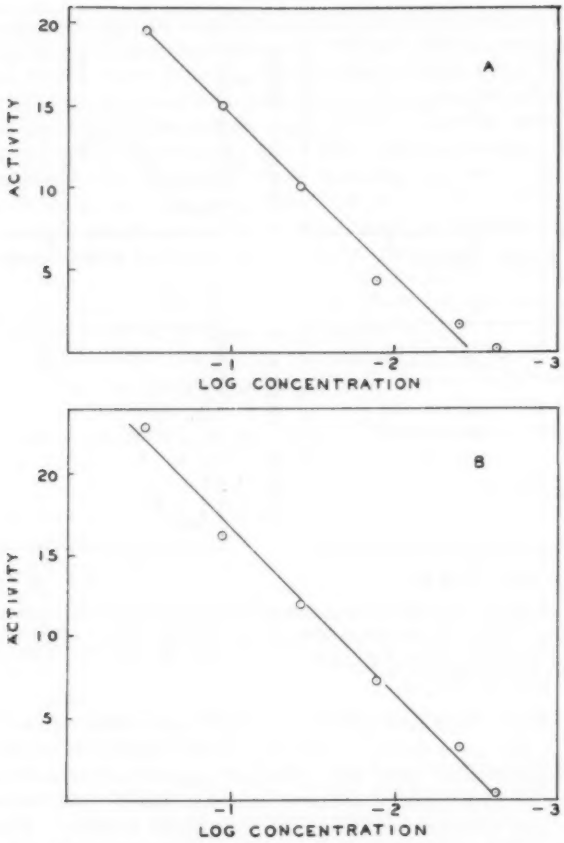

FIGURE 5. Relationship between extract activity on red chromatophores and log of concentration. A. Sinus gland extract. B. Extract of optic ganglia.

to be essentially a simple linear one. The activity was determined in the manner described by Brown and Sandeen (1948) in which the total of all the average chromatophore readings at 15 minutes, 30 minutes, and at 30-minute intervals thereafter until the end of the effect are added together. The difference between this sum and the corresponding sum at the same time intervals which would have indicated no activity was considered a reasonable measure of the activity since it incorporated both the amplitude and duration of effect of the injected extract. Using this dilution-series information to indicate the relationship between the concentration of the principle concerned and the amount of activity, and then using values of activities calculated in a similar manner from the data of Figure 3, it may be estimated that the optic ganglia and brain both contain approximately the same quantity of the principle. The sinus glands appear to possess only about one-half

as much as the brain. The thoracic cord contains about one-thirteenth the amount of the brain, and the connectives only about one-eighty-eighth of the amount in the brain.

It must be borne in mind that these values are still quite rough approximations in view of the calculations being based on the assumption that the amounts of the antagonist (red-concentrating substance) are in equal quantities in all portion of the nervous system, an assumption that is in all probability not justified.

Similarly, values for the relative quantities of red-concentrating principle within the various organs tested assume equal quantities of its antagonist (red-dispersing factor) in all portions.

More precise determination of the quantitative distribution of the two factors within the various tissues must obviously await the development of methods for the complete separation of these from one another.

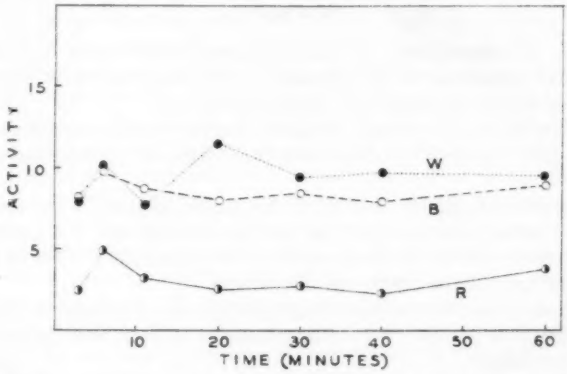

FIGURE 6. Alteration in activity of sinus-gland extracts on red (R), black (B), and white (W) chromatophores over the course of one hour following extraction.

ON THE PROBABLE IDENTITY OF THE BLACK- AND RED-DISPERSING PRINCIPLES

All the data which have been obtained in the course of these experiments upon the red chromatophores support an hypothesis that the red-dispersing principle from the sinus glands and the central nervous system is identical with the black-dispersing principle from the same organs. Two types of experiments have provided particular support.

In the first of these experiments, when eyestalkless assay animals were injected with extracts of various known sources of red-dispersing principle, the sinus glands, thoracic cords, brains, and optic ganglia, extracted in such proportions as to provide concentrations of 1/3, the effects upon the black chromatophores were observed simultaneously with those on the red. As can be seen from the results shown in Figure 3, A, B, D, and E, the action on the red chromatophores parallels in a remarkable fashion the action upon the black after the initial period of retardation. This initial retardation of dispersal has been seen earlier to be due to the simultaneous presence of a red-concentrating factor. Following the retardation the red

pigment usually overtakes the black, for which there has apparently been no similar concentrating principle present, and then the duration of the action of the extracts upon these two pigmentary types is virtually identical.

The second type of experiment involved a determination of the alteration with time of the activity of extracts of the connectives of *Uca* upon three chromatophore types. Extracts of connectives were made up to provide concentrations of 1/3. Five assay animals were injected after 3, 6, 11, 20, 30, 40, and 60 minutes and the activity of the extract determined at each of these times on the red, black, and white pigments. Figure 6 shows the average of the results of three such experiments. The parallel character of the activity of the extracts upon the red and the black chromatophores is conspicuous. In contrast with this, the relationship with respect to the white pigment appears to be different from those of the red and black, and other types of evidence have clearly indicated them to be two distinct substances.

Summary

1. The red chromatophores of *Uca pugilator*, when other factors are equal, exhibit extensive responses to background. They disperse their pigment upon a black background, and concentrate it upon a white one.

2. Under conditions of constant darkness the red chromatophores show a striking persistent daily rhythmicity, dispersing their pigment by day, and concentrating it by night.

3. Extracts of sinus glands, and all of the major portions of the central nervous system, possess strong activity upon the red chromatophores. All portions exhibit two types of activity due to the possession of two principles. One of the activities is red-concentration and the other, red-dispersion.

4. The action of the red-concentrating principle can dominate the response when there is, concurrently present, high concentrations of both red-dispersing and red-concentrating principles.

5. The red-concentrating activity of all extracts is of much shorter duration than is the red-dispersing activity, the latter often lasting 3 to 4 times as long as the former.

6. Evidence is presented which strongly suggests that the black-dispersing and red-dispersing actions are due to one and the same principle.

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THE FATE OF INDIA INK INJECTED INTRACARDIALLY INTO THE OYSTER, OSTREA VIRGINICA GMELIN

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The contributions of Metchnikoff and others to the problem of the role of the phagocyte in the "defense" reactions of invertebrates as well as vertebrates is well summarized in the book, "L'immunité dans les maladies infectieuses" (1901). His concept of the relationship between intracellular digestion in the lower forms of life and phagocytosis in higher forms of organisms still merits the attention of those interested in the evolutionary aspects of the origin of "defense mechanisms." Indeed, the role of the amebocyte in the digestive functions of the oyster has been outlined by several workers (Vonk, Yonge, Takatsuki, Nelson, Chestnut) and its role in "defense" suggested by recent findings of Nelson (personal communication) and Stauber (1945). No careful delineation of the role of the amebocytes [2] of the oyster as a "defense" cell has yet come to the author's attention. Takatsuki's paper on the nature and functions of the amebocytes of *O. edulis* is of real value but stressed the digestive aspects though several pertinent findings of his will be discussed later. On the contrary, the phagocytosis of the gymnospores of the gregarine, *Nematopsis ostrearum* Prytherch, by the amebocytes of the oyster *O. virginica* and the subsequent development of the parasites within these cells is clear evidence of their failure in this case to act as defense cells. This does not constitute proof, however, that the amebocytes are not active in defense since examples might be chosen for citation of phagocytic cells being (1) host cells for parasites (Leishmania in man or hamster, Meleney, 1925; and Sphaerita in commensal protozoa, Wenrich, 1944), (2) disseminators of stages of parasites resistant to digestion (anthrax spores in frog lymphocytes, Trapeznikoff, 1891), (3) host cells for one stage in the life cycle and not for the other stage (cryptozoic *vs.* erythrocytic stages of avian malarias, Huff and Coulston, 1944), or (4) host cells in one anatomical or physiological situation and not in another (zoochlorellae in Tridacna, Yonge, 1944). The cases cited do not constitute proof that intracellular digestion is an impossible achievement in the phagocytic cells of the organisms mentioned.

The use of injected dyes greatly aided investigators in describing the phagocytic cells and their role in local and general inflammatory processes. The papers of Buxton and Torrey (1906) are an especially interesting series of studies on the disposition and fate of metabolizable as well as non-metabolizable particulate matter injected into mammals. Cuenot's report (1914) on the phagocytic organs of molluscs lacks any mention of the oyster. Furthermore, although he used parenteral injections of particulate matter like ink, he was concerned only with their

[1] The author is deeply indebted to Dr. T. C. Nelson for his many suggestions and generous support of this work.

[2] Amebocyte, leucocyte and phagocyte are used interchangeably. The cells under discussion are found free in the blood and are both amoeboid and phagocytic.

immediate removal from the circulation, not their ultimate disposal by the mollusk. Takatsuki (1934) studied the fate of ink and carmine injected into the body of *O. edulis*. His findings, in general, are in agreement with those in the present report. Unfortunately, he fails to include in his report the details for area and amount injected, time and temperature relationships, etc.

Ranson's (1936) findings were based on the exposure of whole living oysters to a sea water medium containing neutral red, or emulsions of aniline oil and other oils or coal tar. Since the absorptive surface in these cases was apparently the whole of the exposed epithelial layer of the oyster, even though he describes a leucocytosis and mass migrations of leucocytes across epithelial layers, his observations are clearly corollary to the general object of the work begun here.

The present study was undertaken as the first step in learning the nature, extent and action of the "defense" processes in the oyster. The results reported herein constitute a clear-cut demonstration of the role of the oyster amebocyte, or phagocyte, in the disposal of large masses of particles of india ink following intracardial injection. Subsequent steps should include studies using similar injections of non-pathogenic and, if possible, pathogenic (for the oyster) organisms as well as the studies of the responses of the oyster to the local parenteral injection of a similar series of particulate matters, non-living and living.

Materials and Methods

A group of oysters was filed down with a wood rasp on the area of the left valve over the pericardium. When the shell had been worn through by the rasping, the pieces of thin shell were picked away with forceps exposing the whole pericardium. This technique was previously found to leave an undamaged oyster (Stauber, 1940) capable of living and functioning for a long time in an aquarium. The filed oysters in this series were held in the aquaria a long enough period for the mantle to lay down a thin prenacreous layer over the area exposed.

On the day of injection the new prenacreous material was picked away. Since preliminary experiments had demonstrated that the heart could not be hit through the intact pericardial wall on every occasion, the pericardium was opened to expose the heart for injection. A dropping pipette with a finely drawn out tip was used for the injections. This was partly filled with a 1 : 10 dilution of Higgins india ink in sea water and inserted into the ventricle of the oyster. Partly by the warmth of the author's fingers and partly by pressure on the bulb, amounts of diluted ink from 0.25 to 0.45 ml. were injected. The rule was to inject as much as possible under the conditions of the experiment. The injection period usually was terminated when the oyster heart became so firmly contracted that further injection of material caused escape of the ink through the puncture wound.

For the type of study reported here and because of the difficulty of estimating the weight of the oyster meat in the intact animal, no attempt was made to quantitate the mass injected on the basis of the body weight of the oyster. Since india ink precipitates in sea water, loose aggregates of various sizes were injected, some macroscopically visible. Every attempt was made to reduce these in size by shaking before injection but often reagglomeration took place in the injection pipette during the period of injection. The question of size of particle injected may be of great importance in the immediate distribution of ink observed and probably further

trials should be run using other materials for injection with more nearly uniform, smaller-sized particles for comparison (see Cuenot, 1914). Though the general picture reported here would be altered somewhat by the particle size introduced, it seems probable that the chief difference would have been an earlier, more widespread distribution of the ink.

After intracardial injection the oysters were returned to aquaria until the appropriate interval had passed (Table 1). They were then opened, the meat carefully shucked out and placed whole in fixative (Zenker-formol 5 per cent) for approximately 15 minutes, then removed to a glass plate and carefully divided into portions by cutting in the transverse plane at right angles to the long axis of the oyster. The pieces were then placed in suitable amounts of fresh fixative for 24 hours before washing. After mounting in paraffin, sections were cut at 7 μ and stained with hematoxylin and eosin.[3]

TABLE I

Record of amounts of ink injected intracardially into the oysters and the length of interval between injection and killing time

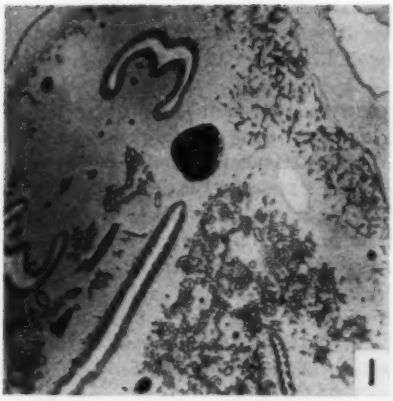
Oyster No.	Am't of india ink injected	Interval between injection and fixation
1	0.25 ml.	15 min.
2	0.3	1 hour
3	0.45	2 hours
4	0.25	4 hours
5	0.4	22 hours
6	0.25	8 days
7	0.25	17 days
8	0.25	25 days
9	0.45	33 days
10	0.25	42 days

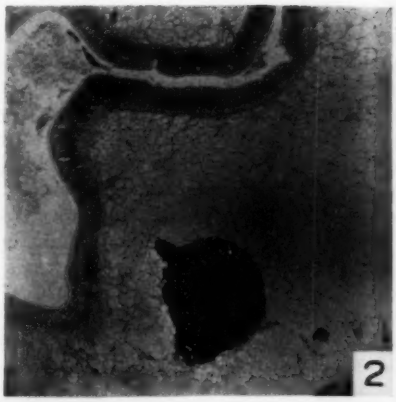
The water temperatures in the aquaria in which the injected oysters were held varied more or less directly with outside temperatures during the period. Since the period covered by the investigation was from April 8 to May 20 water temperatures in the aquaria rose irregularly from 12° to 21° C.

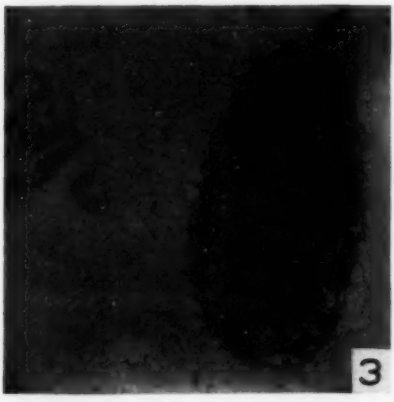
In studying the tissues the several sections of each oyster were studied microscopically, notes being made concerning the distribution of the ink both intracellularly as well as extracellularly, under the medium power as well as under the oil immersion objective. When the whole series had thus been observed key locations were critically reviewed for each oyster. The types of information tabulated may be grouped in three categories: (a) the presence or absence of ink in blood vessels or sinuses of representative areas, (b) migrations of phagocytes containing particles of ink through representative epithelia and (c) presence or absence of ink in various lumina and cavities. The following blood channels were sampled: (1) the ventral pallial artery of the mantle, (2) a sinus of the same area of the mantle, (3) the anterior aorta seen in a typical section through the visceral

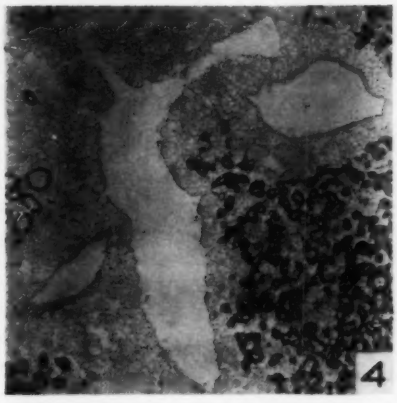
[3] The author is indebted to Miss Deborah Jackson and to Dr. James Shannon, of the Squibb Institute for Medical Research, for preparation of these tissues for microscopic examination.

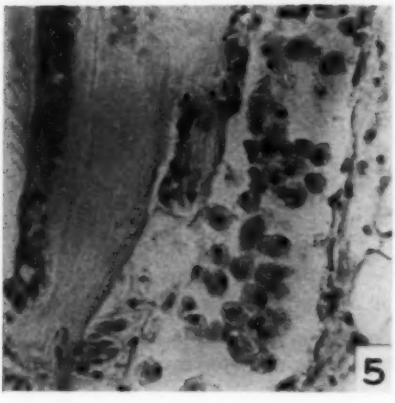
PLATE I

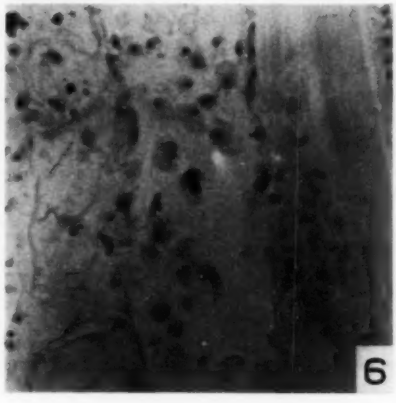












mass, (4) the largest sinus in the visceral mass cross section, (5) a small artery of the visceral mass, a subbranch of one of the hepatic arteries approximately 0.1 mm. in diameter, (6) a small sinus of the same area and magnitude for comparison, (7) an artery in the adductor muscle, (8) one of the posterior gastric arteries in the region of the so-called oral process ventral to the adductor muscle, (9) the heart, (10) the medial gill axis sinus, (11) a lateral gill axis sinus and (12) two vertical gill vessels, one leading from the medial aspect of the inner demibranch toward the medial gill axis sinus and the other from the lateral aspect of the same demibranch to the lateral gill axis sinus. In any area wherever enough phagocytes could be seen (50–100) percentage counts of those containing ink granules were recorded.

Migrations of ink-laden phagocytes through epithelial layers were sought by comparing equal numbers of representative fields from similar areas of the anatomy of the oyster. Except where noted, five consecutive fields were examined for migrating ink-laden phagocytes. The following sites were chosen as representative: (1) mantle epithelium facing the outer palp, (2) outer palp epithelium on side facing the mantle, (3) medial aspect of outer palp facing the adjacent surface of the inner palp on the same side, (4) lining of the promyal chamber near the point where it becomes contiguous with the suprabranchial chamber, (5) lining of the stomach in the area of its greatest diameter, (6) mid gut epithelium in three other locations, two areas in the visceral mass proper, and one in "oral process," (7) epithelium in the rectum dorsal to the adductor muscle, (8) a series of ten cross sections of tubules of the digestive diverticula, (9) gonaducts in region of visceral mass, (10) nephridial tubules, (11) external lining of the heart and (12) internal lining of the pericardium.

Ink, either free or in phagocytes, was sought in the lumina of five portions of the digestive tract mentioned above, in the lumina of the ten tubules of digestive diverticula examined, in the promyal chamber, in the portions of the infrabranchial

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PLATE I

FIGURES 1–6.

FIGURE 1. Oyster No. 3. 2 hrs. after injection. Section through anterior end of visceral mass near the mouth. Anterior aorta and many smaller arteries are occluded with ink. Note absence of ink in large and small sinuses. × 15.

FIGURE 2. Oyster No. 1. 15 min. after injection. Section through tip of visceral mass ventral to the heart. Large and small arteries near the style sac are shown occluded with ink. × 50.

FIGURE 3. Oyster No. 6. 8 days after injection. Section through anterior end of visceral mass. Large aorta occluded by leucocytes laden with ink. × 50.

FIGURE 4. Oyster No. 9. 33 days after injection of ink. Section through anterior end of visceral mass dorsal to palps. Anterior aorta and a large visceral sinus shown. Neither contains any significant numbers of leucocytes. × 50.

FIGURE 5. Oyster No. 4. 4 hours after injection. Section through outer left lamella of outer left demibranch showing a vertical gill vessel. No ink seen in amebocytes; the single dark body in each amebocyte is the nucleus. × 625.

FIGURE 6. Oyster No. 6. 8 days after injection. Section through the inner lamella of the left inner demibranch near the gill axis and flanking the left suprabranchial chamber. A vertical gill vessel is shown containing ink-laden amebocytes. Similar ink-laden cells are also seen extravascularly. × 550.

chamber between palp and mantle and between the palps, in the lumina of nephridial tubules and gonaducts and in the pericardial cavity.

Results

The response of the oyster to the intracardial injection of india ink, under the conditions listed above, may be conveniently divided into four phases: arterial occlusion by the agglomerated masses of ink, phagocytosis of the ink particles, distribution of the ink by the phagocytes and elimination of the ink from the oyster. It is important to note that there was a considerable overlapping in timing of the sequence of these events.

Arterial occlusion. The immediate effect of the intravascular injection of agglomerating particles of india ink is virtual embolism of the arterial system of the oyster. The arterial system as reported by Leenhardt (1926) and Elsey (1935) is clearly outlined in this way. Even while injecting the suspension of ink the anterior and posterior aortae are seen. The arterial branches and sub branches to visceral mass (hepatics and posterior gastrics and anterior ventral), palps, mantle, adductor muscle and rectum are very noticeably blocked in the gross as well as in section with aggregations of free ink particles (Figs. 1, 2 and 9). If the lacunae, or sinuses, in areas near the arteries are examined they are usually found relatively free from blood cells and except for an occasional free granule or two, no ink is to be seen (Fig. 1). The gill axis sinuses are likewise devoid of cells and ink. Other gill vessels may show more blood cells but little or no ink (Fig. 5). The arterial emboli are first composed of ink alone (Fig. 2). From the 22nd hour to 8 days after the injection of the ink the emboli are continued by masses of phagocytes most of which have engulfed particles of ink (Fig. 3). Later, between the 8th and 17th days, with the dispersal of the ink-laden phagocytes the arteries appear as free from cells (Fig. 4) as do those of untreated oysters.

Phagocytosis. No unquestioned phagocytosis of ink was noted at the 15 min. interval (oyster No. 1) though it would easily have been obscured in the ink-filled arteries. Little or no phagocytosis was noted anywhere before the fourth hour (oyster No. 4). Occasionally, as in the gill axis vessels of oyster No. 3 at 2 hours, where numerous blood cells were seen, 1–2 per cent contained a few granules of ink. Elsewhere in the same oyster phagocytes with ink were even rarer.

Beginning with oyster No. 4 more phagocytes were seen in the arteries and more contained ink, though the distribution may be variable. For example, there were many phagocytes containing ink in arteries in the adductor muscle or visceral mass but few with ink in the ventral pallial artery.

By the end of the first day after injection (oyster No. 5) mass accumulation of phagocytes in the arteries was observed. Furthermore, almost all the ink is intracellular in those phagocytes which continue the embolic obstruction of the vessels, with most of the phagocytes containing ink granules (see Fig. 3, oyster No. 6 for a similar condition). In direct contrast to this picture, although blood cells are seen in vertical and gill axis sinuses or sinuses of viscera and mantle, the percentage of phagocytes containing ink particles ranges from 2 per cent in a lateral

gill axis and a small sinus of the visceral mass of this oyster (No. 5) to 15 per cent and 19 per cent in vertical gill sinuses.

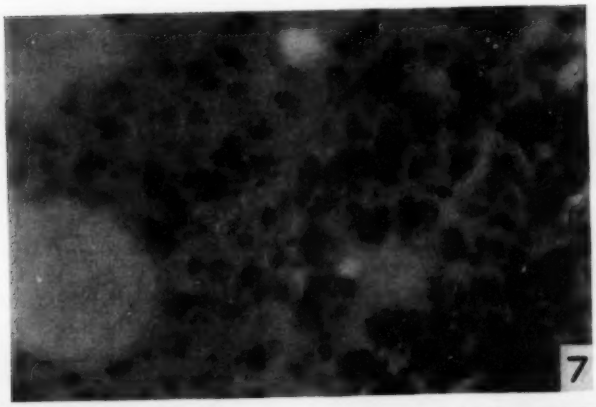
Subsequently ink is only occasionally seen free; the granules are observed almost entirely in the cytoplasm of the phagocytes. The presence of a few ink granules in blood vessels throughout the later period (oysters No. 9 and No. 10) is possibly due to its release from phagocytes upon their death or through "defecation" by active phagocytes (Chestnut, thesis), although there is no direct evidence to support this.

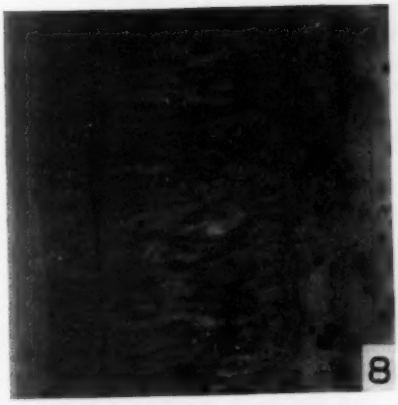
The origin of the phagocytes which engulf the large amount of ink injected is an important point to be considered. It should first be noted that even in the untreated oyster "the origin and mode of production of amebocytes are not known; no definite organ is known to produce them" (Takatsuki, 1934). It is well known and easily observed that there is a large mobile reserve of phagocytic cells. Although few of these are seen within the arteries or even in the larger blood sinuses, many are found in the smaller sinuses of gill, mantle and viscera. Probably most, however, are scattered in the smallest lacunae or intercellularly especially under the epithelium of the digestive tract, digestive diverticula and gonaducts. Their role in digestion has already been referred to and numerous migrations of phagocytic cells in the epithelia can easily be noted in oysters (Fig. 9).

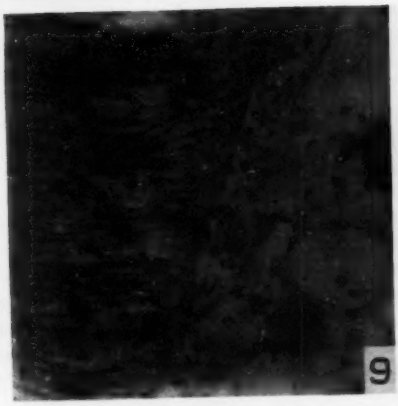
It is unlikely that mitosis of pre-existing phagocytes plays a predominate role in producing (or mobilizing) the phagocytes which dispose of the ink since, of the many phagocytes examined, only one has been observed in mitosis. This is very similar to the role of hematogenous lymphocytes and to a lesser extent monocytes as described by Maximow (1927) for the local inflammatory process in the subcutaneous tissues of the rat injected with trypan blue (Taliaferro, 1949). Since careful study of the arterial walls does not disclose mass migrations of phagocytic cells through them from the surrounding tissues their arrival at the ink masses via the hematologic route is presumed. This is understandable since, although obstruction of the artery in Figure 2 is complete, this is in an oyster *after* fixation and shrinkage. The shrinkage observed is believed not to be merely a function of the fixative used as found by Orton (1937) in his study of the effects of Bouin's fixative on isolated 0.1 gm. pieces of oyster tissues, but also of the blood loss of the cut oyster. Thus, it seems probable that blood flow did not altogether cease and that the slight trickle of fluid through these almost occluded channels made possible the appearance of the phagocytes in the ink-filled areas of the vessels. An alternative, acceptable explanation might be the migration of the phagocytes to the ink-filled arteries by their own activity. This would be possible even in a direction counter to the current (incomplete occlusion) if the current were weak,[4] and if the phagocytes moved along the blood spaces using the lining as a substrate for amoeboid activity. The usual picture of finding blood cells almost exclusively in such a position in the larger vessels may be offered as evidence, though Cuenot believed cells similarly placed on the walls of the small arteries of the "liver" of the amphineuran, *Acanthocites discrepans* to be fixed phagocytes. In the case of the oyster their relative inconstancy of number and position mark them as free phagocytes. The net result by either means would be the arrival in the blocked areas of large numbers of phagocytic cells.

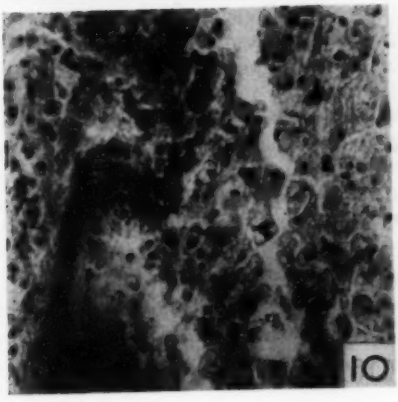
[4] The studies of T. C. Nelson (personal communication) and Hopkins (1934) show much reversal of current, much surging back and forth of blood in the vessels, especially in the mantle.

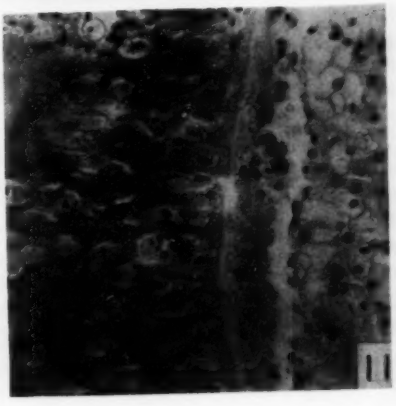
PLATE II











Takatsuki discusses the types of blood cells seen in *O. edulis* and recognizes two main types: granular leucocytes and hyaline leucocytes. No data are available on the proportionate numbers of these two types of cells in oysters or on the relative phagocytic properties of the two types of cells. Indeed, confirmation of his findings is desirable since the hyaline leucocytes are claimed to be non-nucleate and not observable in stained preparations. Since the granules in the granular leucocytes are not stained by hematoxylin-eosin the relative importance of the two cells in phagocytosis is not known, except that the only figures in Takatsuki's report which certainly identify the cell type and also show ingested particles are granular leucocytes.

Distribution. Ink-laden phagocytes become distributed throughout the oyster following engulfment in the arteries. This is accomplished in two ways: first, by the passage of the cells from small arteries to sinuses in the usual fashion, and second, by direct migration through the walls of the blocked arteries both large and small. Both processes proceed actively. For example, the large increase in the proportion of ink-laden phagocytes in the gill axis sinuses (from 2–19 per cent in oyster No. 5 to 35–50 per cent in oyster No. 6) is indicative of the first process and Figure 7 clearly shows the migration of ink-laden phagocytes through the wall of a hepatic artery in the viscera of oyster No. 6. This phenomenon was first seen in oyster No. 5 (22 hours). It was characteristic of the findings for oysters No. 6, No. 7 and No. 8 even though occlusion of the arteries was no longer to be observed in oysters No. 7 and No. 8. Some migrations were still to be observed in oyster No. 9 but the process of migration through arterial walls was virtually completed between the 33rd and 42nd days.

The net result of these processes is the widespread distribution of ink-laden phagocytes so that wherever phagocytes are found in oysters after 8 days in this series, ink-laden specimens can be observed. This is in marked contrast to the distribution of ink in a mammal, like the rat, after intracardial injection. In the mammal the ink largely remains in the organs with macrophages strategically placed to remove material from the blood (spleen, liver, bone marrow).

PLATE II

FIGURES 7–11

FIGURE 7. Oyster No. 6. 8 days after injection. Section through wall of branch artery in visceral mass. Large numbers of ink-laden phagocytes seen in the lumen of the artery as well as many seen migrating through the wall of the artery. × 760.

FIGURE 8. Oyster No. 9. 33 days after injection. Section through wall of style sac showing migrations of ink-laden phagocytes. × 550 approx.

FIGURE 9. Oyster No. 1. 15 min. after injection. Section through style sac in approximately the same region as in Figure 8. Note the presence of a few amebocytes migrating through the epithelium of the style sac. Note also the artery occluded with ink. × 550.

FIGURE 10. Oyster No. 7. 17 days after injection. Section through palp region. To left is medial aspect of outer palp; to right is that portion of the infrabranchial chamber between the faces of inner and outer palps on the right side. Note the many ink-laden amebocytes on either side of the epithelium but especially in the infrabranchial chamber. × 600.

FIGURE 11. Oyster No. 9. 33 days after injection. Section through epithelium of intestine in the region of the oral process. Note the numerous ink-laden phagocytes migrating toward the lumen of this portion of the alimentary tract. × 550.

Takatsuki observed a similar widespread distribution of carmine-laden phagocytes in oysters injected into the body with that substance.

Elimination. The normal physiological movement of amebocytes through various epithelia is well known especially with reference to digestion (Yonge, Nelson, Takatsuki). It is not surprising then to find large scale migration of ink-laden phagocytes across epithelia into various lumina. Table II summarizes these findings. Note first that no migration of phagocytes containing ink was found until more than 22 hours had elapsed after injection of the ink. The first migrating forms with ink in them were seen in oyster No. 6 killed 8 days after injection and subsequent to the first migrations through the arteries. The epithelia of digestive

TABLE II

Migrations of ink-laden phagocytes through epithelia after intracardial injection of india ink

Oyster No.	Time after injection of ink	Number of ink-laden phagocytes observed in the epithelium†								
		Mantle, facing palp*	Palp, facing mantle*	Palp, facing palp*	Lining of promyal chamber*	Stomach*	Intestine visceral area*	Pericardial wall	Rectum*	Digestive diverticula**
1	15 min.	0	0	0	0	0	0	0	—	0
2	1 hour	0	0	0	0	0	0	0	—	0
3	2 hours	0	0	0	0	0	0	1	0	0
4	4 hours	0	0	0	0	0	0	0	0	0
5	22 hours	0	0	0	0	0	0	—	0	0
6	8 days	2	0	6	1	0	4	15	—	0
7	17 days	1	1	5	0	8	6	41	0	14
8	25 days	1	0	1	2	0	0	—	1	1
9	33 days	0	1	7	1	36	33	1	15	10
10	42 days	0	0	2	0***	20	8	6	2	9

— Area not observed.
0 No migrations observed.
* 5 oil immersion fields studied.
** 10 cross sections of tubules of digestive diverticula examined.
*** Many ink-laden phagocytes seen in the promyal chamber.
† The numbers are relative and of chief significance when compared with others in the same vertical column.

organs (stomach, intestine, rectum, digestive diverticula) were the chief sites of the migrations (Figs. 8 and 11) though the presence of many ink-laden phagocytes between the palps on one side of oyster No. 7 (Fig. 10) or in the promyal chamber of oyster No. 10 indicates that the process occurred in a wide variety of epithelial sites. Migrating phagocytes were observed in the lining of both the pericardial wall and the outer aspect of the heart as well as in the pericardial space between these sites.

There were three sites in this series which, almost without exception, showed no migrating ink-laden phagocytes. These were the epithelia of the external (shell-secreting) face of the mantle, the gonaducts and the excretory tubules. Migrations through the epithelium of the gills were relatively rare though it is possible that this is a reflection of the initial distribution of the ink. While the primary occlusion

of the arteries in the visceral mass, and the later migrations of ink-laden cells through the arterial walls of the visceral arteries, would account in part for the sites of migration observed, the failure to observe such migrations through gonaducts, excretory tubules and the external face of the mantle must be explained in some other fashion. Similarly, the presence of 35–50 per cent ink-laden phagocytes in the gill sinuses of oyster No. 6 is contrasted to the rare migrations of these cells across the gill epithelium. Takatsuki reported similar migrations of india ink or carmine-laden phagocytes across epithelia in various sites after injection of carmine into the body of the oyster, but his findings included migrations into excretory tubules and gonaducts. The discrepancy is not easily explained with the data available. Even his possible faulty interpretation through the use of the term "excretion" to include the voiding of undigested material from the body cannot explain the migrating leucocytes he observed. Nowhere in his report does he record the site of the injection of the carmine or ink. If the site had been into or in the vicinity of the excretory organ, a plausible explanation would be available; although, in this series, microscopic fields have been observed showing many ink-laden phagocytic cells just beneath the basement membrane of the epithelium of the nephridial organ without a single phagocyte in migration through the epithelium. Perhaps the frequency of migrations unrecorded by him through these sites is a partial explanation. In the series reported here, only a single ink-laden cell was observed traversing the epithelium of an excretory tubule. The absence of phagocytes from the lumen of the excretory tubules might be explained by the open pericardium since the wall of the auricle is claimed to have excretory functions (Ranson, 1936). This is especially true since we did observe ink-laden phagocytes in the pericardial cavity. In the case of the gonaduct, Takatsuki himself was at loss to explain such migrations. It is possible that his study was made in the autumn when the oyster amebocytes play an important physiological role in the involution of the gonad. Our study covered a period in the spring when the gonad was showing renewed activity. No migrations into gonaducts were observed.

The net result of these migrations was the voiding of ink into the lumina of organs leading to the outside. In fact, once the ink particles had passed through these epithelia they may be considered essentially as being "outside" of the body of the host. It is possible, of course, that some of these phagocytes (especially in the intestine and digestive diverticula and even on palp and gill surface) may migrate back into the oyster again if the ink present in their cytoplasms does not interfere with the performance of other functions. A net loss of ink would be expected, however, which would eventually lead to complete elimination.

Further evidence of this elimination was seen in the appearance in the aquaria of dejecta and rejecta of much darker appearance than that of uninjected oysters under similar conditions. Since no microscopic observations were made on these voided masses it cannot be stated whether the granules of ink were free or still in phagocytic cells when voided from the oyster. Chestnut (personal communication), after local parenteral injections of small amounts of india ink, states definitely that the ink in the feces was in phagocytes.

It will be noted from Table II that ink was still being eliminated from the oyster when the experiment was terminated, though the peak of elimination occurred on or about the 33rd day after the injection of ink. Oyster No. 10 was noted as having

less ink present than any other oyster in the series. Although there are no supporting data available it is believed that the process would continue for a relatively long period of time with the curve of elimination gradually flattening out and with some ink still present, possibly for a year or more.

Discussion

The process of elimination of india ink after intracardial injection is not confined to the oyster. Although no references to exactly similar work in mollusca have been found, vestiges of a similar mechanism for the removal of foreign bodies are described for mammals. Two related statements from Maximow and Bloom, "Textbook of Histology" (1939), are pertinent. Discussing free macrophages of the blood the authors state, "Many investigators have described macrophages in the blood. In animals injected intravenously with vital dyes or corpuscular matter, such as blood cells, large quantities of these substances accumulate in the free macrophages. They originate in the spleen, liver and bone marrow from fixed macrophages through contraction and isolation. They are found especially in the blood of the veins and of the right heart and the major part of them is filtered off in the capillaries of the lungs, but some may occasionally enter the general circulation" (p. 43). "If the macrophages have taken up indigestible particulate matter, as colloidal silver or india ink, they often degenerate; then the foreign particles are set free and may be taken up again by other macrophages. In the intestines and in the lungs the foreign particles may be eliminated from the body with the cells containing them" (p. 91). A local response of similar nature is described for the lungs. In this case, part of the carbon inhaled in city air is phagocytized and expelled in the so-called "dust cell," a macrophage of the lung. In the oyster, however, the process is more closely related to the intracellular phases of the digestive process.

Table III

Time sequence of events leading to disposal of india ink after intracardial injection into the oyster

Oyster number	Time after injection	Arterial occlusion	Presence of free india ink in blood vessels	Migrations of ink-laden phagocytes through arterial walls	Migrations of ink-laden phagocytes through epithelia
1	15 min.	+	++++	−	−
2	1 hour	+	++++	−	−
3	2 hours	+	++++	−	−
4	4 hours	+	+++	−	−
5	22 hours	+	+	+	−
6	8 days	+	±	++	+
7	17 days	−	±	++	++
8	25 days	−	±	++	++
9	33 days	−	±	+	++++
10	42 days	−	±	−	++

The numbers of pluses are approximate and relative and are significant only when compared with others in the same vertical column.

In a poikilothermic animal, like the oyster, the rates of various metabolic processes are greatly influenced by temperature. That the degree of activity of the phagocytes would be similarly affected by temperature is a logical conclusion. Therefore, the times reported here for phagocytosis, distribution and elimination of india ink (see Table III) must be considered in terms of the temperatures prevailing during the experiment. If the experiment had been conducted at 25° C. the events noted most probably would have occurred much sooner than recorded above.

A half century ago the responses of many organisms to the injection of foreign bodies, living and dead, were studied but the oyster was not included. It is strange that the oyster, an organism of such great value as a food in many parts of the world, especially in this country, and claimed to be scientifically the best known marine animal, has not been fully studied in this respect. Only a fraction of the oyster eggs fertilized each year grow to adult oysters. Many causes of mortality are known, predators like the starfish and oyster drill being especially important. There are always found, however, losses of unknown causation (Orton, 1924; and Loosanoff and Engle, 1941). Except for Tennent's work on *Bucephalus*, Prytherch's work on *Nematopsis* and Korringa's recent report on a fungal shell disease, few diseases peculiar to the oyster are known where the parasitic organism (not predator) produces direct effects on the living substance of the oyster (Dollfus, 1921; Orton, 1937). This is not meant to imply that *Bucephalus* or *Nematopsis* is necessarily a primary cause of mortality of the oyster. It is very unlikely that these are the only ones that do exist. The beginning study reported here is believed fundamental to a full knowledge of host responses upon which control measures might be based.

A similar series of oysters should be studied using foreign body particles of much smaller size. While arterial occlusion would probably be lacking and initial distribution of the ink less restricted, the phenomena of phagocytosis, migration and elimination would most likely be similar.

In the life cycle of *Nematopsis ostrearum*, a gregarine parasite the sporozoite of which normally develops in the phagocytes of the oyster, Prytherch notes that "though some of the sporozoites are evidently destroyed by the phagocytes a small number generally survive and grow rapidly at their expense." The mortality of sporozoites in a light natural infection was estimated at better than 50 per cent. Development of the sporozoite and spore formation in the oyster require about two weeks in warm weather, according to Prytherch. It is entirely possible that, in addition to the mortality mentioned above, there is an important sporozoite loss due, not to intracellular digestion by the phagocytes, but to migration and elimination of the sporozoite-laden phagocytes as in the case of the india ink granules described above. Indeed, unless the hypertrophy of the sporozoite-infected phagocyte restricts amoeboid activity, it is possible that the elimination of developed spores in this fashion may constitute the more normal route of infection for the crab host.

Summary

The responses of the oyster to an intracardial injection of a sea water suspension of india ink were followed grossly and microscopically. The ink suspensions agglomerated readily and produced emboli which virtually occluded the arterial vessels of viscera, mantle and adductor muscle. Subsequent events, with considerable over-

lapping, were in sequence: (a) phagocytosis of the injected ink particles by mobile phagocytes, (b) distribution of the ink in the phagocytic amebocytes to all parts of the organism with concomitant resolution of the emboli and (c) eventual elimination of the ink from the organism by the migration of ink-laden phagocytes through the epithelial layers of the alimentary tract, digestive diverticula, palps, mantle, heart and pericardium into lumina from which they were voided. The epithelia of gonaducts, nephridia and shell-forming mantle were not routes of migration. A close relationship is noted between the role of the phagocytes in the normal digestive process and in the "defense" reaction to such a foreign body as india ink. The possible significance of the responses noted with respect to unexplained mortalities of the oyster is considered.

The author is deeply indebted to Dr. T. C. Nelson for taking Figures 2–4 and 7–11 inclusive.

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MULTICONJUGATION IN BLEPHARISMA

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Conjugation of three or more individuals, i.e. "multiconjugation," has been observed in mass cultures of *Blepharisma undulans*. The phenomenon in this species was first reported by Giese (1938a), and has been seen in a variety of other ciliates during the course of the last century (Chen, 1946). Chen, working on *Paramecium bursaria*, published the first detailed cytological study of multiconjugation. In the present paper an analogous cytological study is described for *B. undulans*, and experiments are set forth indicating how multiconjugation may be brought about.

Two stocks of *B. undulans* have been employed, one from the Laboratory at Woods Hole, the other from Elon College, North Carolina. Both of these are morphologically equivalent to Giese's California stock (1938a; also below). The organisms have been cultured at room temperature, with filtered rain water as medium. A rich supply of bacterial food could be maintained in mass cultures by adding a barley seed to each culture dish. For cytological work, given animals have been put on slides, fixed in Carnoy, dried, and stained with methyl green without counterstain (Weisz, 1950).

MULTICONJUGATION

In dense cultures, conjugation epidemics have been observed at intervals, occurring almost simultaneously in different culture dishes and involving perhaps 90 per cent or more of all animals present. Rough counts have shown that approximately 5 per cent to 10 per cent of all matings seen were conjugations of three individuals. Occasionally four, and rarely five, individuals have been found joined into multiconjugant systems. All other conjugations have been paired matings.

Examination of a large number of "triples" has revealed two principal patterns of attachment among conjugants. In one pattern, the three participants are fused together firmly along the full extent of their peristome areas, i.e., from near the anterior tip of the animals almost to mid-level. Attachment is equally extensive between any two of the three members, and the trio suggests a tripod opened up in three dimensions. In another pattern, two of the three conjugants (A and B) are firmly and fully fused as above and as in paired mating, while the third conjugant (C) is peristomially attached to a small area of the peristome of either A or B. In these cases any direct physical connection is not evident between conjugant C and one of the other partners (cf. photographs by Giese, 1938a). Attachment of conjugant C to the posterior body regions of either A or B, as observed by Chen (1946) for *P. bursaria*, has not been seen in *B. undulans*.

In cases of matings involving more than three animals, a large variety of attachment patterns has been noted. Conjugant A might be joined to B, C to A, D to C

and/or A, E (if present) to B, or to C, D, or A, etc. Attachment varies in extent but it is always peristomial. It would, therefore, appear generally that conjugative fusion in *B. undulans* always takes place along the peristome area, and that effective fusion need involve only a very small section of this area.

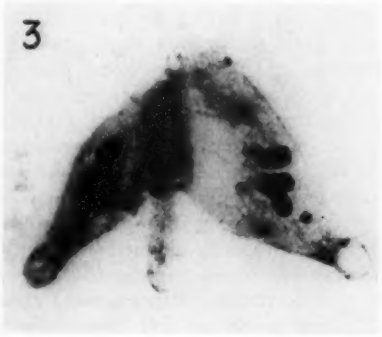

PLATE I

FIGURES 1–4

FIGURE 1. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Animals are joined tripod-fashion. Soon after initial fusion; macronuclear chains beginning to contract. Appr. times 200.

FIGURE 2. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Left individual joined less extensively. Macronuclear contraction under way. Appr. times 200.

FIGURE 3. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Animals joined tripod fashion. After synkaryon formation; in each member two macronuclear primordia visible. Note "kernspalt"-like appearance of primordia. The smaller primordium in each member will degenerate. Appr. times 200.

FIGURE 4. Conjugation of 3 animals, prepared slide, Carnoy-Methyl Green. Animals joined tripod fashion. Late stage of conjugation. In each member only one presumptive macronucleus left; the latter in process of elongation. Appr. times 200.

Multiconjugation typically occurs among animals of equal size, although in many cases one (or more) of the conjugants is smaller than the others. Conjugative fusion lasts as long as in paired mating, i.e., approximately 3 days. After this time the participants separate in groups or singly within a few hours. All the exconjugants are viable and produce vigorous clones.

Cytological

Young (1939) has described in detail the essential cytological steps in normal conjugation of the Yale race of *B. undulans*. The Woods Hole and Carolina stocks differ somewhat from the Yale strain, particularly in the structure and the vegetative behavior of the macronucleus (Weisz, 1949), but the sequence of processes during conjugation is virtually identical to that in the Yale race (Weisz, 1950). The old macronucleus first condenses, fragments, and then degenerates, disappearing completely approximately 36 hours after the conjugants become fused initially. A synkaryon has formed in the meantime, and numerous readily visible, relatively large micronuclei now begin to appear. Simultaneously a number (typically four) of faintly staining macronuclear primordia arise. One of these enlarges, takes up an increasingly dense stain, passes through a "kernspalt"-like stage (Weisz, 1950), and ultimately becomes the definitive macronucleus of a new caryonide. The other primordia degenerate and disappear. During this phase the micronuclei become smaller, and throughout the remainder of the caryonide life history micronuclei are extremely small and often cannot be found at all. It is largely due to this circumstance that synkarya, and pregamic and postzygotic maturation stages cannot be reliably identified. The derivation of micronuclei and of macronuclear primordia from the synkaryon can, however, be inferred by experiment; experimentation has similarly brought out the temporal correlation between micronuclear and the visible macronuclear events (Weisz, 1950). Thus, for any given stage of the macronucleus, the micronuclear condition at that stage can be estimated. It should be noted parenthetically that in typical *B. undulans* only one macronuclear primordium persists, so that a caryonide is defined by the entire clone arising from an exconjugant.

In multiconjugation involving any number of conjugants, characteristic nuclear changes have been consistently observed in every one of the participating animals. This has been found true regardless of the specific pattern of peristomial attachment. The changes are always precisely identical in nature and duration to the cytological sequence observed in paired mating, and they occur synchronously in all members of a multiconjugant system. This is illustrated in Plate I, showing successive stages in the conjugation of three individuals. Since the old macronuclei disappear and new primordia develop, as in paired mating, it may be inferred that synkarya are probably formed in each of the members of a multiconjugant system. In this respect *B. undulans* and *P. bursaria* (Chen, 1946) are similar.

Experimental

In order to determine whether or not multiconjugation is attributable to the existence of multiple mating types, twenty-five single-clone cultures (including both Woods Hole and Carolina stock animals) have been prepared, and individuals from each clone have been mixed with individuals from every other clone. Animals from

one of the clones to be tested have first been exposed to light for 1 to 3 days, a procedure which brings out depigmentation of the animals; organisms from another clone were left in the dark, ensuring the retention of the pigmented state (Giese, 1938b). Some 100 animals from each of the two clones have then been transferred together to a deep watch glass containing fresh, relatively food-free culture medium. Under such conditions the members of the two clones can be identified; moreover, transfer to new medium effects a fairly sudden reduction of food organisms, a condition necessary to induce conjugation (Giese, 1938a).

The mixing of clones in Blepharisma, unlike similar procedures in Paramecium, does not result in a striking mating reaction. Nevertheless, after some 20 to 50 hours more than 90 per cent of all animals present are involved in the mating process. This has been found true for mixtures of any two of the twenty-five clones studied. The result confirms similar observations of Giese (1938a). Inspection of each of the mixtures has shown that the majority of conjugation pairs consisted of members from only one of the clones, i.e., in perhaps 60 per cent to 70 per cent of the pairs both conjugants were either deeply pigmented or only lightly pigmented (some restitution of pigment having taken place in the interval). In the remaining mating pairs conjugants of different clones have been involved. In each of the denser mixtures, moreover, a number of multiconjugant systems has been found. Most of these consisted of three animals, usually derived from only one clone. Occasionally, however, four-animal and five-animal conjugations have been seen, and in some of these one or two of the participants derived from the second clone.

In a variation of these tests, organisms from a single clone have been transferred alone to fresh, food-free culture medium. As above, 1 to 2 days after transfer the great majority of animals present have been found in conjugation, and in the denser test cultures a number of multiconjugations could be observed.

Thus it is likely that both the Woods Hole and the Carolina stocks of *B. undulans* are selfing strains; that physiological differentiation into distinct mating types is absent, and that the occurrence of multiconjugation (as of paired conjugation) is primarily a function of food supply and of population density. The age of a clone does not appear to play a decisive role, since conjugation has been induced as early as in the 6th- to 10th-generation descendants of exconjugants.

Discussion

A question of primary interest concerns the basic significance of the observed nuclear changes in multiconjugation. In one of the characteristic patterns in which conjugant A is extensively joined to conjugant B, and C is attached less extensively to B, does conjugant B exchange pronuclei with both A and C? From the manner of attachment it might appear probable that A and B exchange pronuclei as in paired mating, while an autogamous process takes place in conjugant C.

Unfortunately, evidence for the occurrence of autogamy in Blepharisma cannot, at present, be adduced by genetic means (as Sonneborn, 1939, has done for *P. aurelia*), in the absence of mating types, or of other demonstrably gene-controlled characteristics. Moreover, clear-cut cytological evidence for (or against) autogamy is, to date, not available for *B. undulans*, and this is not surprising in view of the inadequacy of the micronuclei for cytological investigation. Macronuclear events are always identical in all conjugative processes, and thus cannot be relied upon

to differentiate between autogamy (if it occurs) and true cross-fertilization. Conceivably, an autogamous process need not necessarily be postulated for the second major pattern of multiconjugation, in which A, B, and C are joined in tripod-fashion, the extent of attachment being mutually equivalent among the members. In these cases a pattern of pronuclear exchange might occur, involving all three participants; but the possibility of autogamy is by no means ruled out. In multiconjugations involving four and five individuals, attachment of some of the members is often exceedingly insecure and superficial; these members separate off upon slight agitation very soon after they are initially joined, yet normal conjugative macronuclear changes have been observed in a number of such separated individuals. For these, at least, there probably can be no question of nuclear exchange.

Thus, although direct proof is lacking, the very occurrence of multiconjugation in *B. undulans* probably provides presumptive evidence for the occurrence of autogamy. If so, multiconjugation in this species is cytologically wholly equivalent to this type of "mating" in *P. bursaria* (Chen, 1946).

Summary

A cytological and experimental study was carried out on conjugations involving more than two individuals of *Blepharisma undulans*. Attachment of the participants in a conjugating group occurs in a number of characteristic patterns. Regardless of the attachment pattern, nuclear changes occur in all members of a multiconjugant system. These changes are identical in nature and duration to those observed in normal paired conjugation, and occur synchronously in all participants of the system. The stocks of *B. undulans* used are found to be selfing, physiologically not differentiated into mating types. Multiconjugation can be induced by varying food supply and population density. The probability of autogamous processes in connection with multiconjugation is discussed.

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THE EFFECT OF X-RADIATIONS ON SQUID LARVAL TROPISMS

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The eye of the squid, *Loligo pealli*, is structurally very much like that of the higher vertebrates except that the sensory cells of the retina are directly exposed to the light source (Fig. 1). The eyes are among the first organs to differentiate, and the squid embryos manifest positive phototropism and negative geotropism long before the time of hatching. The chromatophores of the squid embryo are relatively very large, and appear and function at a very early stage. It seemed of value, therefore, to determine the effect of various x-radiation exposures on the sensory responses of the squid embryo and larva as evidenced by the tropistic responses and chromatophores.

Materials and Method

The squid eggs and early embryos were procured by the Supply Department of the Marine Biological Laboratory in the form of clusters of long strings, and were brought immediately to the laboratory where they were kept in running sea water. In general, all eggs or embryos in a single "finger" of the string mass are in the same stage of development. This is less apt to be the case as development progresses, because the effect of the slight differential in oxygen supply will become increasingly evident. The stages used in this series were, however, early (pre-hatching) and were essentially similar in respect to the stage of development for each set of observations.

The embryos [2] were exposed to the radiations while still within their capsules and jelly strings. They were placed in 40 cc. of fresh sea water in celluloid containers. These were circular De Witt Bait boxes, called Pyrashell, measuring 65 mm. in diameter and 16 mm. deep. The controls were kept under identical conditions during the irradiation of the experimentals. Immediately after irradiation, the embryos (both experimentals and controls) were transferred to regular finger bowls, each of which contained 100 cc. of fresh sea water, and suspended in a water bath kept at a constant temperature of 20° C.

The x-radiation conditions were as follows. The radiation equipment used in this series was the standard equipment of the Marine Biological Laboratory, Woods Hole, Mass. This consists of a pair of General Electric Coolidge tubes, water cooled, operated at 185 kvp and at 30 ma. At the low exposures one tube was

[1] This document is based on work performed under Contract AT-30-1-Gen-70, for the Atomic Energy Commission.

The author acknowledges, with appreciation, the technical assistance of Mrs. Lona Barthelmes.

[2] For convenience the term "embryo" refers to the pre-hatching and "larva" to the post-hatching stage, regardless of how hatching is achieved.

PLATE I

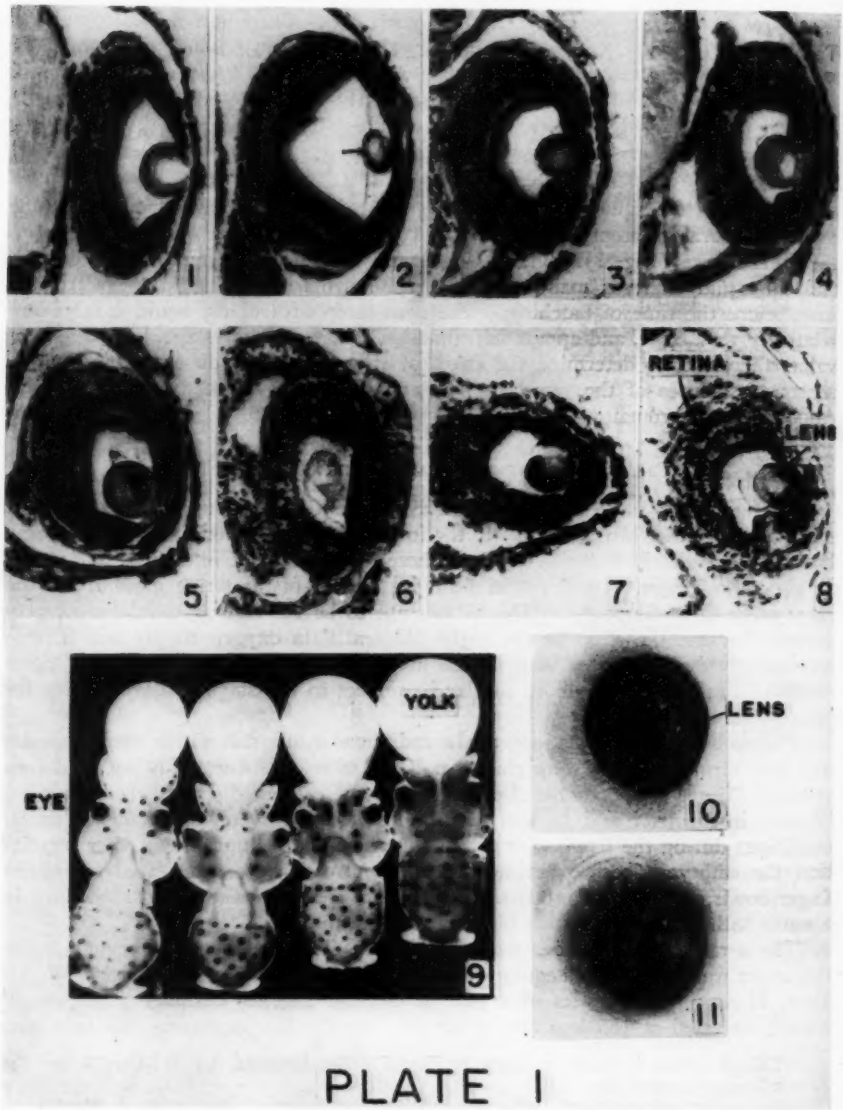

PLATE I

blocked out and the distance was such that 30 roentgens were delivered to the specimens per minute. For the doses above 1000 r the two tubes were used, without filters, at such a distance that the dose rate was 2720 r per minute. X-ray exposures ranged from 50 r to 200,000 r in single exposures.

Observations and Experimental Data

There were three immediate and pronounced physiological effects of high dosage x-ray exposure of the early squid embryo, namely a loss of the positive phototropism and the negative geotropism, and the rupture of the jelly capsule, liberating the embryo.

Tropisms. Prior to x-irradiation, all hatched squid embryos will swim upward (negative geotropism) and toward any source of light (positive phototropism). These inevitable reactions are present for the unhatched but motile embryos as can be demonstrated by artificially (surgically) liberating them from their capsules. The phototropism is first manifested when the eyes become structurally differentiated, and the negative geotropism becomes evident when the embryonic siphon begins to function, causing the embryo to move vigorously. The weight of the embryo, with its large yolk sac (Fig. 9), normally causes it to sink, except that this negative geotropistic response tends to keep the embryo bobbing up toward the surface where, no doubt, it acquires a more abundant supply of oxygen.

Low dosage x-irradiation has very little effect on the tropistic responses. Only when the exposures reach 10,000 r or more is there any obvious (statistical) variation in responses. At this dosage level less than 50 per cent of the free-swimming larvae will move toward the light. At exposures of from 1000 r to 5000 r only stray larvae were seen swimming about aimlessly, with no evidence of tropistic direction. Not until 100,000 r is reached do the embryos appear totally indifferent to any source of light.

Histologically one cannot find any immediate change in the retina or lens which might be correlated with the functional tropistic changes. The radiation effect is

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Plate I

Figures 1–11

Figure 1. Section through eye of control squid embryo at same age as Figures 2 through 19, and identical with left specimen in Figure 9. (Compare with other control photographs, Figures 12 and 13.)

Figure 2. Section through eye of squid embryo four days after the embryo received 50 r x-rays.

Figure 3. Same as Figure 2 except exposed to 100 r x-rays.

Figure 4. Same as Figure 2 except exposed to 200 r x-rays.

Figure 5. Same as Figure 2 except exposed to 400 r x-rays.

Figure 6. Same as Figure 2 except exposed to 800 r x-rays.

Figure 7. Same as Figure 2 except exposed to 20,000 r x-rays.

Figure 8. Same as Figure 2 except exposed to 50,000 r x-rays.

Figure 9. Four squid embryos prior to hatching, three of which were exposed four days previously to x-irradiation. From left to right: Control, 1000 r, 5000 r, 50,000 r. Note persistence of embryonic chromatophores and yolk mass, and stunting of the irradiated specimens.

Figure 10. Entire eye of control (from Figure 9) showing large normal lens.

Figure 11. Entire eye of squid embryo which received 100,000 r x-rays four days previously. Entire eye small, with retina and lens smaller than control.

PLATE II

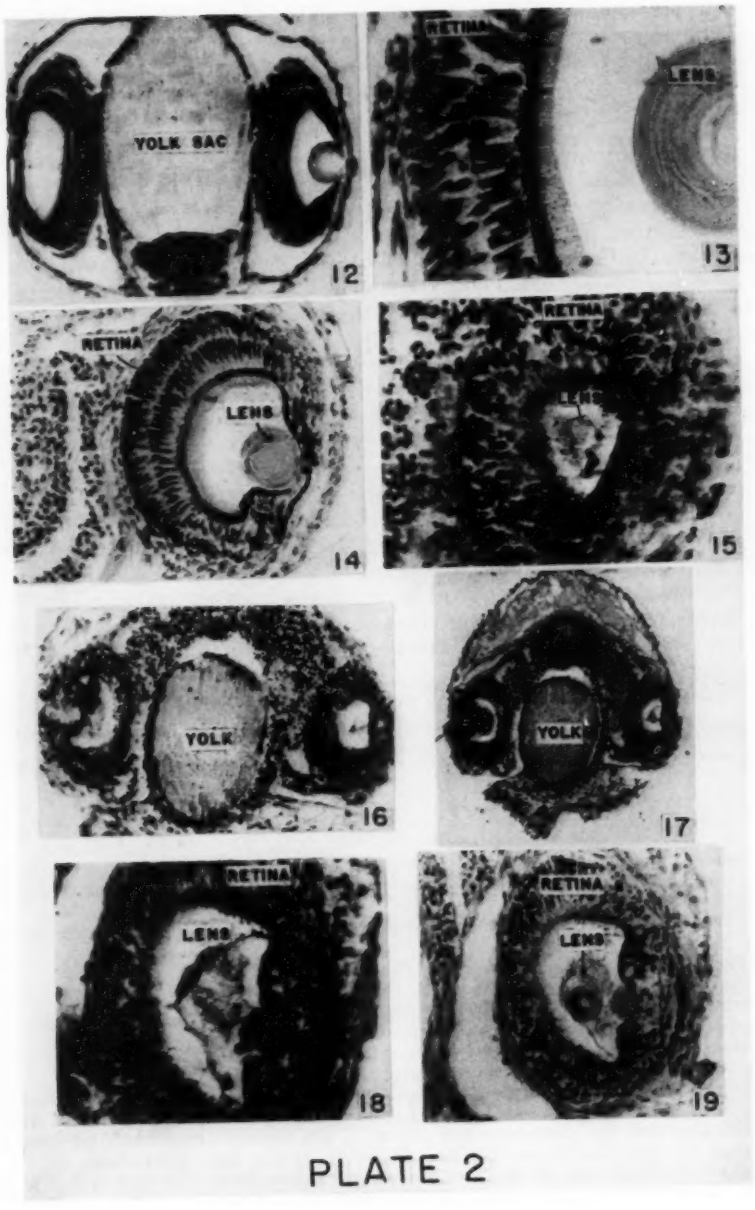

PLATE 2

immediate upon the behavior of the embryo but is delayed with respect to observed histological changes in the optic system, as discussed below.

The negative geotropistic response is probably due to the nervous system as a whole, not particularly to the optic system. It is not so easily altered. By 50,000 r the embryos swim about aimlessly (with regard to the gravitational field) settling on the bottom of the container where their expected survival is poor because, presumably, there is insufficient oxygen to maintain the ontogenetic changes that are ensuing. Since, in contrast with the eye as the seat of phototropic responses, there is no known sensory center for geotropistic responses, one can only analyze the behavior of the embryo with respect to the forces of gravity.

High dose x-irradiations do not immediately kill the early embryo of the squid even though the tropistic responses are altered. Some embryos survive 200,000 r for as much as 120 hours.

Chromatophores. The chromatophores of the early squid embryo are few in number but they are very large, relative to the size of the embryo as a whole (Fig. 9). They are also very active, long before the time of hatching. There is no experimental evidence as to their control as there is for fish chromatophores (Parker, 1940; Tavolga and Rugh, 1947; and Tavolga, 1949). X-irradiation at any level used (50 r to 200,000 r) had no effect on the contractility of the chromatophores although it slightly altered the rate, accelerating their contractions and expansions. It did, however, stop the growth of the chromatophores as of the time of high dose x-irradiation, so that such embryos remained small and relatively dark as compared with the controls which grew larger, and paler. This effect is readily apparent after 3 or 4 days when there is sufficient ontogenetic change in the controls with which to compare the x-irradiated embryos (Fig. 9). The persistence of the functioning embryonic chromatophores following x-irradiation has been demonstrated for other embryos (Rugh, 1949). The chromatophores continue to function, so that the neuromuscular corollaries are unaltered, as long as the embryo is alive. The elements which control the chromatophores and the tropistic responses are therefore probably different.

PLATE II

FIGURES 12–19

(Note: Figures 12, 16, 17 at same magnification. Figures 14 and 19 at same magnification. Figures 15 and 18 at same magnification. Figure 13 at twice the magnification of 15 and 18.)

FIGURE 12. Section through bilateral eyes of squid embryo at termination of experiment, showing normal relation of optic cup, lens, and "corneal" epithelium. Compare with Figures 16 and 17.

FIGURE 13. High power magnification of Figure 12 control, showing highly developed retina and lens of the squid.

FIGURE 14. Squid embryo exposed to 100 r x-rays, showing slight eye damage after 4 days. General topography of the eye parts unchanged, but epithelium slightly irregular.

FIGURE 15. Disorganized retina and lens 4 days following x-irradiation to 800 r.

FIGURE 16. Section through bilateral eyes of living and functional squid embryo 4 days following x-irradiation to 20,000 r. Note extensive necrosis.

FIGURE 17. Same as Figure 16 except x-irradiated to 50,000 r. Note retardation in development (size).

FIGURE 18. Disorganized retina and lens 4 days after exposure to 50,000 r x-rays. Note many pycnotic nuclei.

FIGURE 19. Same as Figure 18 except that embryo was exposed to 100,000 r x-rays.

Hatching. The hatching process of the squid embryo requires the rupture of the thick jelly capsule and the subsequent emergence of the free-swimming embryo or larva. Normally this hatching process is due to the elaboration of a hatching enzyme, as in the case of fish (Armstrong, 1938) and the amphibia. At low x-ray exposures (i.e., 50 r to 1000 r) there is little induction of hatching, but as the dose approaches 50,000 r nearly 100 per cent of all embryos will be artificially hatched, even though they may not have been developmentally prepared for it. This premature hatching is due to a swelling and bursting of the jelly membrane around the embryo. This swelling of the jelly membrane, due probably to imbibition, appears far in excess of any parallel change in the embryo itself, possibly because of the high water content of the jelly. This phenomenon of the capsule was demonstrated by Evans, Beams and Smith in 1941 for the Arbacia egg. It is most graphically seen around the very early egg (i.e. cleavage stage) when the space between the membrane and the egg is almost doubled by high exposure to x-irradiation. Such eggs are, of course, immotile and are not generally liberated as are the active embryos of later development.

Muscular Activity. The early squid embryo consists of a large yolk sac which is surrounded by a thin muscular membrane (envelope) connected with the differentiating organs of the embryo proper (Fig. 9). As the yolk is consumed the embryo grows until the reduced yolk sac is gradually surrounded by the growing squid tissues. All during this process there is a constant, rhythmic contraction of the muscular envelope surrounding the yolk. These contractions vary only with the general environmental temperature and the activity of the embryo. When exposed to high doses of x-radiation there is only a slight acceleration of the contraction rate. Even at the very high exposures of 200,000 r these rhythmic smooth muscle contractions were not particularly affected, and were seen to persist long after the loss of the tropistic responses and until just before the embryo succumbed.

Histological Effects. There is always a latent period in the histological manifestation of x-irradiation damage (Rugh, 1949). The squid embryo is no exception, but since it differentiates rather slowly, it is not until 72 to 120 hours after exposure that one can observe significant changes in the retina and lens of the eye, for instance. All tissues are affected, but the greatest damage appears to be on the optico-ocular system and since this is presumed to be the system responsible for the phototropism, our description will be limited to the retina and the lens.

As previously stated, the eye of the squid is morphologically far advanced for an invertebrate organism and its visual acuity is evident from a period long before hatching (Figs. 1, 12, 13). The histological corollaries of loss of tropistic reactions are not evident for 3 to 4 days after x-irradiation at which time the retina of the 100 r or 200 r organism shows only slight disorganization (Figs. 14, 4), and the retina of the 800 r or more organisms shows extensive disorganization (Figs. 6–8, 15–19). The nuclei and cells of the control retina are arranged in concentric layers. While the x-irradiated eye at 800 r (or more) retains its spherical shape (Figs. 10, 11) the retinal layers lose their regularity and the lens becomes aspherical, and its fibers become disarranged (Figs. 15–19). There is no growth following x-irradiation so that the eye mass remains small (Figs. 10, 11). The histological changes can be seen readily by comparing Figures 1 through 8, all of which were magnified to the same degree, and all were from specimens fixed 4 days fol-

lowing x-irradiation of the experimentals. All were alive and active at the time of fixation. The extensive necrosis and pycnosis, even at 50,000 r and 100,000 r, were apparently not sufficient to kill the organisms (Figs. 18, 19).

Summary and Conclusions

1. The pre-hatched squid embryo can tolerate x-irradiation exposures to at least 200,000 r without succumbing for 4 days.

2. X-irradiation of the average squid embryo, within its jelly, to 25,000 r will liberate it from its capsule; while 50,000 r induces 100 per cent hatching. Irradiation-induced hatching is brought about by a swelling of the jelly and a bursting of the capsule, allowing the liberation of the motile larva.

3. The strong phototropistic response of all recently hatched squid larvae is immediately nullified in many specimens following x-irradiation to 10,000 r, but 100,000 r completely nullifies this response in all larvae, although they remain motile.

4. The strong negative geotropism of the squid larva is immediately reduced by 50,000 r and completely nullified by 100,000 r. This effect is also immediate, but the larvae remain motile.

5. Growth of squid larvae is stopped immediately by x-irradiation to 5,000 r or more, but no dose used (up to 200,000 r) had any immediate effect on the functioning of the smooth muscles, so that the motility of the organism as a whole was unaffected. The chromatophores and eyes remained the size they were at the time of x-irradiation.

6. The histological effects of x-irradiation were most damaging to the retina and lens of the eye, but were not manifest for 3 to 4 days. The retina showed extensive disorganization within 4 days following 800 r (or more) but since larvae lost their tropistic responses immediately (following high exposures) it must be postulated that long before these latent histological effects were evident, the retina was functionally disconnected from the motor organs which directed the normal organism toward the light source.

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THE ELECTROCARDIOGRAM OF ARENICOLA

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There is evidence, on the basis of the accelerating action of acetylcholine, that the hearts of Arenicola and Lumbricus are neurogenic (Prosser and Zimmerman, 1943). Nerve cells, which may be pacemakers, are present in the hearts of Arenicola (Carlson, 1908). Another test of the nature of the pacemaker is the electrocardiogram (ECG) which in myogenic hearts (vertebrates and molluscs) consists of regular slow potentials and in neurogenic hearts (Limulus, decapod Crustacea, some insects) is oscillatory. The electrocardiogram of arthropods may, in addition, have slow components but the oscillations appear to correspond with the repetitive discharge of the pacemaker neurons (see Prosser *et al.*, 1950, for references). The electrocardiographic test of the pacemaker has not been applied to annelids and, in fact, no electrocardiograms of annelids have been published.

Electrocardiograms of Arenicola were recorded with an Offner amplifier and crystograph; the amplifier was capacity coupled and of variable time constants, the longest being 0.2 seconds. Electrodes were of silver-silver chloride either in direct contact with the tissue or connected by cotton wicks soaked in sea water. Efforts to record electrocardiograms from Lumbricus and Neanthes were unsuccessful but extensive records were obtained from the hearts of six large specimens of *Arenicola cristata*. The worms were anesthetized with chloretone, the two hearts were exposed and recovery from the anesthesia was permitted in running sea water as described by Prosser and Zimmerman (1943). One worm was pinned to a paraffin block and the hearts exposed without anesthesia, but excessive body movement resulted in an unsatisfactory preparation. Usually an indifferent electrode was placed on the liver or inner body wall and an active electrode was put on different parts of a heart, from the lateral gastric vessel down to the ventral end. There was much interference with electrocardiograms by slow potentials arising from movements of the intestine and muscles of the body wall, and it was necessary to make many records from each animal with varying electrode positions, several time constants and different states of recovery from anesthesia to obtain a consistent picture of the electrocardiogram.

Several typical records are shown in Figure 1. The electrocardiogram (ECG) of *Arenicola cristata* consists of an initial rapid negative wave followed by a positive deflection, often with several superimposed oscillations (1a, 1b, 1c, 1e, 1f). The number, duration and shape of the oscillations are variable and they usually terminate with a sharp negative spike (1c, 1e) which may be followed by a slow negative wave (1d, 1f). The precise form varies in the same locus from time to time and from one region to another; such variability is more characteristic of oscillatory than of slow-wave types of electrocardiogram. If slow potentials had been present, the amplifier would have detected them as shown by the slow potentials recorded

from hearts of squid and mussel (Fig. 2) and the many interfering slow potentials from body wall and intestinal muscle.

In Arenicola the beat normally starts in the lateral gastric vessel and spreads ventrally over the heart. Records taken from the dorsal portion (lateral gastric vessel) and from middle and ventral regions were qualitatively similar and showed no greater differences than did records from the same region at different times during a two-hour period of observation. Similar records were obtained whether wicks or wires made contact and electrode movement was so slight that it did not introduce an artifact. Regular electrocardiograms were occasionally observed when there was no visible contraction.

FIGURE 1. Electrocardiograms from Arenicola. Monopolar recording. In all records except 1a an upward deflection indicates negativity of the heart lead. 1a and 1b from same animal, all others different individuals. Time mark 1 second. In 1a three downward deflections between electrocardiograms were caused by activity of somatic musculature. Long time constant for records 1d and 1f, short time constants for other records. Note oscillatory spikes in 1a, 1b, 1c and 1f.

It was not possible to obtain a mechanical record of the contraction simultaneously with the electrical record. However, the heart was closely observed and the dilation and systole marked on the moving recording paper. The fast, initial component of the ECG consistently occurred before or simultaneously with dilation and the systole occurred later, often during the slow wave. Whether the fast potential is an action potential associated with active dilation, or whether there is a comparatively long delay between the action potential and systolic contraction is not certain, but the latter alternative seems more likely.

For comparison with Arenicola the electrocardiograms of known neurogenic hearts (Limulus and the spider crab Libinia) and known myogenic hearts (Loligo

and a fresh-water mussel Amblema) are given in Figure 2. The records from Limulus (Fig. 2a, b) resemble string galvanometer records (Garrey, 1932) and the one from Libinia (Fig. 2c) resembles records from Astacus (Hoffman, 1911), while the molluscan electrocardiograms (Figs. 2d, e, f) resemble records from Octopus (Luisada, 1932) and Ostrea (Taylor and Walzl, 1941). The electrocardiograms of Arenicola do not have the persistent oscillations of the arthropods but they do show several variable spikes and lack the large regular slow waves of molluscs and vertebrates. In one preparation a long series of spikes appeared without any visible beat; such irregular spikes are sometimes found in records from Limulus hearts.

FIGURE 2. Electrocardiograms from neurogenic and myogenic hearts. Monopolar recording, upward deflection indicates negativity of active lead. 2a, c, d, e long time constant, 2b short time constant. Time records 1 second. a, b, Limulus. c, spider crab Libinia. d, e, fresh-water mussel Amblema. f, gill heart of squid Loligo. In 2d three vertical lines indicate point of systole. Note oscillatory spikes in 2a, 2b, 2c and presence of slow waves only in 2d, e and f.

It appears, therefore, that the electrocardiogram of Arenicola consists of summated spike potentials which may correspond to discharges from a ganglionic pacemaker. The fast components may be followed by a slow potential, the nature of which is as uncertain as it is in arthropod hearts. The electrocardiogram supports the hypothesis that the heart of Arenicola is neurogenic.

SUMMARY

The electrocardiogram of *Arenicola cristata* consists of a rapid negative deflection followed by a positive deflection, often with superimposed oscillations and often followed by a slow negative wave. There is much variability from animal to animal and from time to time at the same position.

The oscillatory nature of the electrocardiogram supports the hypothesis that the heart of Arenicola is neurogenic.

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DUALITY IN THE ELECTRICAL RESPONSE OF THE LATERAL EYE OF LIMULUS POLYPHEMUS

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Photoreceptor activity is accompanied by electrical changes, and the lateral eye of Limulus is no exception. Illumination of this photoreceptor produces a transient change in voltage (Graham, 1932; Hartline, 1928; Hartline and Graham, 1932). This change in voltage, called the retinal action potential, is measurable across the eye, front to back, and its polarity is such that the corneal surface becomes transiently negative to the back of the eye.

The retinal action potential recorded from the lateral eye of Limulus in response to a short period of illumination (Graham, 1932) exhibits a rather simple contour, consisting of a monophasic negative wave which returns to the base line. Close inspection of this response (Fig. 2, Graham, 1932) reveals a small step-like initial elevation which precedes in time the larger negative wave. Heretofore, no study, nor even any mention, has been made of this initial phase of the retinal action potential of the lateral eye of Limulus. The experiments which are the subject of this report describe some of the properties of this initial phase and indicate its possible source.

Materials and Methods

A rectangular piece of the carapace, with the lateral eye in the center, was cut out and dissected free from the underlying tissue. This section of the carapace was mounted in a lucite electrode chamber with a low melting point wax, which also covered all of the outer surface of the carapace, except the cornea. The corneal surface of the eye was bathed by sea water in the forward compartment, in the bottom of which there was a silver-silver chloride electrode. The back of the eye was bathed by sea water in the rear compartment which also contained a silver-silver chloride electrode. The chamber was then covered and placed in the path of a light beam in an otherwise light-tight compartment, and the silver-silver chloride electrodes were connected to the grids of the amplifier. One hour in the dark was found to be sufficient for complete dark-adaptation.

The light source was a 500 watt tungsten filament projection bulb. The light beam passed through 8 cm. of water and a heat absorbing filter. The intensity of the light beam was controlled by Wratten neutral tint filters. The maximum intensity incident on the cornea was 27,000 foot candles, which was considered as unit intensity. The duration (0.01 sec.) of the light flash was controlled by a photographic shutter. A portion of the light beam activated a photocell, the output of which was recorded as the stimulus signal. The potentials were fed into high-gain, condenser-coupled amplifiers and recorded on an ink writer. The frequency

[1] Lalor Fellow, Summer, 1949, during which period a large part of the work reported was completed.

response of the ink-writer is flat to 100 cps. The amplifiers were operated at different time constants, in order to accentuate the duality of the response. The nominal time constants were calibrated with a square wave voltage pulse.

After dark adaptation of the excised eye, a flash of light of the lowest intensity was admitted and the response recorded. Ten minutes later a second flash of light was admitted and so on until the desired intensity range was covered. When a second series was desirable, an hour was again permitted for completion of dark adaptation and the sequence was repeated.

The effect of light-adaptation upon the electrical response of the excised eye was determined by admitting a test flash of light 5 seconds after the end of a controlled period of exposure to light. The intensity of and exposure to the adapting light could be readily varied.

A third experimental procedure consisted of the introduction of substances into the sea water bathing the eye. These materials were introduced under dim ruby-red light. Recovery from the exposure to red light was permitted from 2 to 7

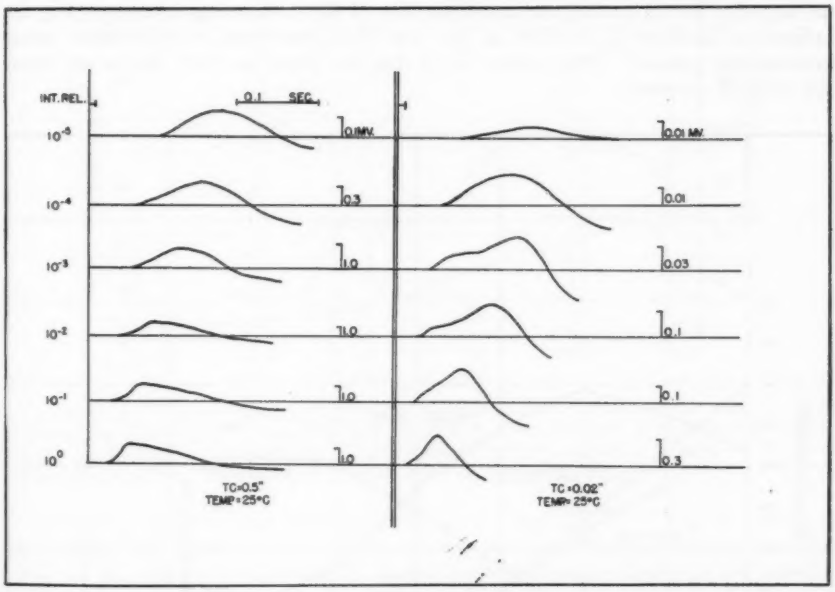

FIGURE 1. Protocols of typical experiments with the lateral eye of Limulus. The original records were reenforced with black ink for photographic reproduction.

Left. A series of responses elicited by test flashes of 0.01″ duration (indicated in the upper line only) and of intensities (10° represents 27,000 foot candles) indicated in the left hand column (int. rel.) opposite the appropriate records. These responses were recorded with 0.5″ time constant. Upward deflection indicates negativity of the corneal electrode. Calibration signal (millivolts) follows each record. The time base is indicated above the upper record.

Right. A similar series of responses recorded with a short time constant (0.02″) to emphasize the dual nature of the response. Exposure, intensity, calibration and time base as above.

minutes, depending upon the intensity of the next test flash. In control experiments, the exposure to red light did not affect the magnitude or contour of the response to a test flash administered immediately after the exposure to the red light, presumably because the intensity of the test flash was sufficiently high.

Forty-eight experiments were conducted at room temperature, which varied from experiment to experiment in the range 25° to 28° C. The crystograph pens recorded with red ink, which had to be reenforced with black india ink for photographic reproduction.

Results

1. Results obtained with 0.5" time constant

The responses recorded from an excised Limulus lateral eye, stimulated with light flashes of different intensities, exhibit a rather uniform contour and a progressive increase in magnitude (a typical series is illustrated in Fig. 1, left). The relation of retinal action potential magnitude (measured from base line to peak of the negative wave) to the common logarithm of the stimulating intensity (Fig. 2) indicates a tendency to level-off at low and high intensities, with a rather linear intermediate portion. The latency of the response decreases with increasing intensity (Fig. 2, crosses).

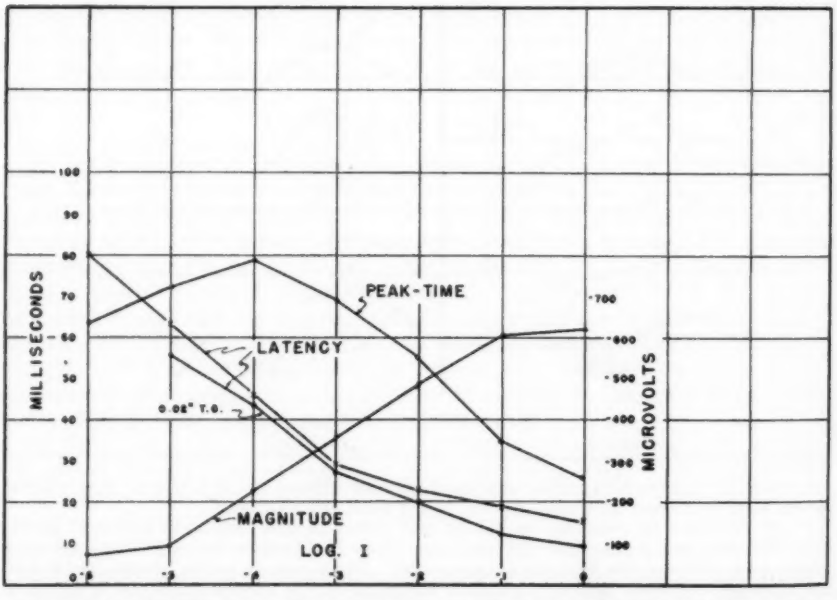

Figure 2. Graphs relating latency, peak-time and magnitude of the retinal electric response to the logarithm (base 10) of the stimulating light intensity. Log 0 represents an intensity of 27,000 foot candles. All data were obtained from responses recorded with 0.5" time constant, except latency curve marked 0.02" T. C.

2. Results obtained with 0.02" time constant

These responses were obtained in a manner similar to the above and a typical series from one experiment is illustrated in Figure 1, right. The presence of two phases in the electrical response to flash intensities of 1×10^{-3} and greater is apparent. From such records and others not shown, in which the resolution of the two phases was even greater (Fig. 4, left, lower line), it was possible to measure the magnitudes of the two phases. The relation of these magnitudes to the common logarithm of the flash intensity is shown in Figure 3 (1, magnitude of the first phase; 2, magnitude of the second phase). The departure of this magnitude contour from the more conventional one of Figure 2 may be attributed to the short time constant, as discussed below.

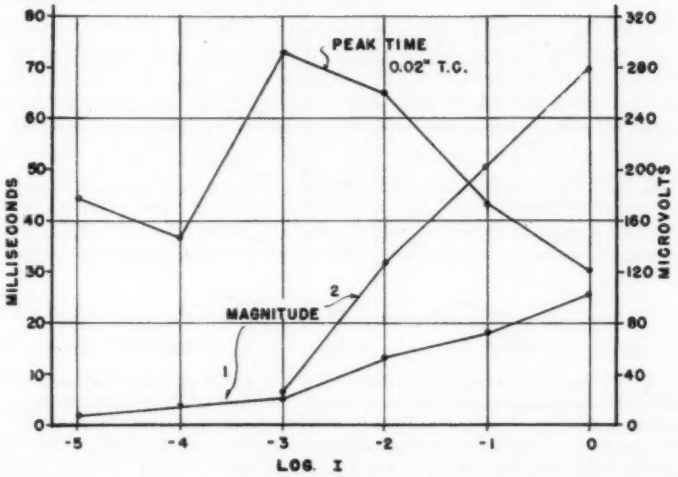

FIGURE 3. Graphs relating peak-time and magnitude of the retinal electric response to the common logarithm of the stimulating light intensity. These data are from responses recorded with a short (0.02") time constant. The magnitude relation marked 1 pertains to the initial phase; that marked 2 pertains to the second phase of the retinal response.

The responses to flash intensities of 1×10^{-4} and 1×10^{-5} (Fig. 1, right) are of uniform contour and do not indicate two phases. The gradual regression, in time, of the second phase (Fig. 1, right) from the lower record on up, suggests another measurement; namely, the time required for the response to reach maximum, hereafter called peak-time. Peak-time is measured from the beginning of the response to its maximum. The relation of peak-time to the common logarithm of the intensity is illustrated in Figure 3 (short time constant) and in Figure 2 (longer time constant).

The latency of the initial phase of the retinal action potential decreases progressively with increasing intensity, giving the relation shown in Figure 2 (0.02" T. C.).

3. Effect of light adaptation

The effect of light adaptation on the retinal electric response, using the short time constant, is illustrated in Figure 4, left. Within the particular conditions of this experiment, it is apparent that light adaptation of 200 foot-candle seconds renders the initial phase invisible and causes some reduction of the magnitude of the second phase. These effects are quite reversible, as indicated in the control record, obtained 10 minutes after the preceding rsponse.

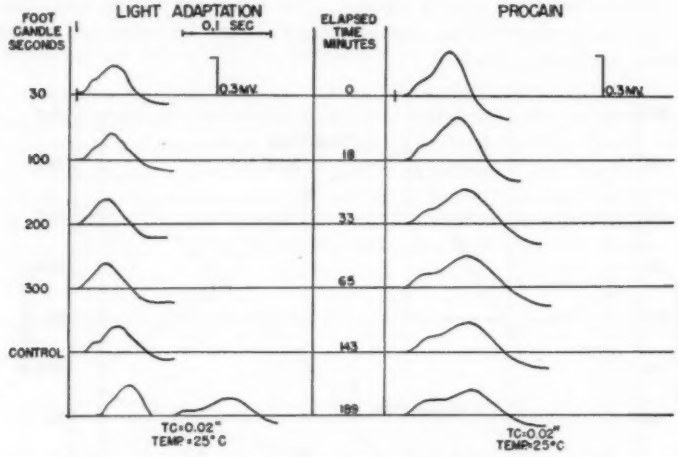

FIGURE 4. Protocols of experiments illustrating the effect of light adaptation (left) and procain (right) on the retinal electric response of the Limulus lateral eye elicited by a constant intensity, constant duration test flash (270 f. c., 0.01"). Column marked "foot candle seconds" indicates the exposure to which the eye was subjected preceding the test flash, which was administered 5 seconds after the adapting light was cut off. Column marked "elapsed time minutes" indicates time elapsed (eye in dark) after the drug was administered. Lower line of left column illustrates the extremes of dissociation of the two phases encountered during these experiments. Amplification and paper speed were constant throughout and are indicated on upper line.

4. The effect of procain hydrochloride

The addition of procain hydrochloride to the sea water in the rear compartment of the eye chamber, in amounts to bring the final concentration to 0.02 per cent, produced immediate cessation of optic nerve activity and effected a gradual reduction in the magnitude of the second phase of the retinal response (Fig. 4, right). The magnitude of the initial phase is but little affected, even in responses (not shown) recorded six hours after introduction of the drug.

5. Variability of dissociation of the two phases

The extent to which the two phases of the retinal action potential are summated varies somewhat from preparation to preparation (Fig. 4, left column, lowest line).

The dissociation of the phases in any one preparation was rather constant throughout. The presence or absence of the rudimentary eye (the mass of white pigment behind the lateral eye) did not affect the two phases of the retinal action potential in any way.

Discussion and Conclusions

1. Is the double response an artifact?

a) Excised lateral eye preparations kept for 8 hours at room temperature generally showed the onset of deterioration by a gradual reduction in magnitude of response. Both phases of the retinal action potential were affected. Preparations kept at room temperature for 20 hours often were totally unresponsive. Apparently both the initial and the second phase are characteristic of living eyes.

b) The dual response has been recorded with silver-silver chloride electrodes and with salt bridges leading to calomel half-cells. The dual response is also apparent in published records (Fig. 2 of Graham, 1932) obtained with an entirely different amplifying and recording system. The duality of the electrical response cannot be considered an artifact attributable to the recording electrode system.

c) The initial phase disappears upon light adaptation (Fig. 4, left) and reappears upon dark adaptation. The initial phase appears in time after a characteristic latency which varies with the intensity of the incident illumination (Fig. 2). These facts indicate that the duality of the retinal action potential is characteristic of the functional photoreceptor.

2. Which phase persists at low intensities?

Measurements of the time from the onset to the crest of the retinal action potential (peak-time) plotted as a function of the log intensity (Fig. 3) reveal a marked discontinuity between log $I = -3$ and log $I = -4$. This indicates that the crest of the response to a flash intensity of 10^{-4} occurs earlier in time than the crest of the response to a flash intensity of 10^{-3}, which is apparent also in Figure 1, right column. Scanning of these records from bottom to top indicates that the crests of the responses to flash intensities of 10^{0} to 10^{-3} are the crests of the second phase. At 10^{-4} only one phase is present and, since the crest occurs earlier in time, it is implied that this is the crest of the initial phase. The second phase is apparently absent at this and lower intensities.

A similar conclusion follows from consideration of the magnitude-log intensity relationships of Figure 3. The magnitude of the second phase (2) decreases rapidly from 10^{0} to 10^{-3}. Extrapolation of this curve would indicate a complete absence of the second phase in response to flash intensities of 10^{-4} or lower. It must be suggested, however, that recording with a short time constant sacrifices a large part of the transient voltage change, particularly that associated with the second phase. This undoubtedly produces some distortion of the magnitude-log I relation (compare with a similar curve, Fig. 2). The initial phase, on the other hand, shows a gradual increase in magnitude and the maximum at log $I = 0$ is appreciably less than the magnitude of the second phase. Such data were obtained from six experiments where the degree of dissociation was sufficient to make these measurements possible throughout the entire intensity range.

3. The significance of the two phases

The rapid disappearance of the initial phase upon light adaptation, coupled with the fact that low intensity light flashes elicit only the initial phase, suggests that this response may be associated with receptor cells of greater sensitivity than those which give rise to the second phase. This consideration implies that the lateral eye of Limulus contains two functionally different kinds of sense cells. It is of great interest that two structurally different kinds of sense cells have been described for the lateral eye of Limulus (Demoll, 1914). The complex eye of Limulus is com-

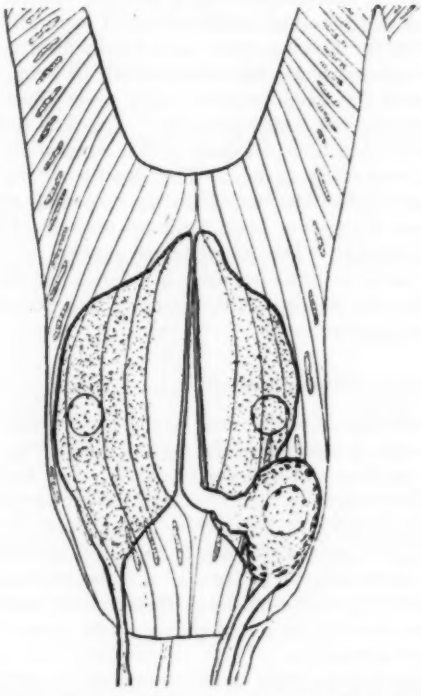

FIGURE 5. Diagrammatic representation of a longitudinal section through an ommatidium from the lateral eye of Limulus showing the radial retinal cells and the eccentric retinal cell with its central process, all embedded in an epithelial framework. (From Demoll, 1914.)

prised of many visual units, the ommatidia. All ommatidia, according to Demoll (1914), possess a similar spatial arrangement of receptor cells (Fig. 5). There are a group (10–15) of receptor cells radially arranged about the longitudinal axis of the ommatidium, leaving a hollow core in the center. This core is partially occluded by a process originating from an eccentrically located sense cell (Fig. 5). In each ommatidium there is only one eccentric sense cell and this seldom contains pigment, whereas the radially located sense cells often contain pigment (Demoll,

1914). The relative paucity of the eccentric sense cells, their centrally located processes and their freedom from pigment makes them the likely source of the initial phase of the retinal action potential. Further, in certain experiments the magnitude of the second phase of the retinal action potential has exceeded 1 millivolt, whereas the initial phase rarely exceeded 0.1 millivolt. This response magnitude ratio of 10 to 1 roughly parallels the population ratio of radially arranged sense cells to eccentric sense cells, 10—15 to 1. It is concluded, therefore, that the response of the eccentric sense cells to illumination produces the initial phase and the response of the radial sense cells produces the second phase of the retinal action potential.

The presence of two morphologically and physiologically different types of receptor elements in the lateral eye of Limulus is reminiscent of the dual nature of most vertebrate retinae.

Summary

Retinal action potentials recorded from the excised lateral eyes of Limulus, using a short time constant amplifier coupling, show a dissociation into two discrete responses, an initial and a secondary phase, over part of the intensity range. Analysis of the data indicates that the single response present at low intensities of stimulation is the initial phase and that both phases are present only in response to higher intensities of illumination. This sequence of single and dual phases with increasing intensity of stimulation gives rise to the peculiar peak-time relation shown in Figure 3. Light adaptation causes a rapid disappearance of the initial phase and only a gradual disappearance of the second phase. The magnitude of the initial phase is much smaller than the magnitude of the second phase. These data suggest that the initial phase represents the response of highly sensitive receptors (eccentric retinula cells) that are outnumbered by less sensitive receptors which contribute to the second phase (the radial retinula cells) (Demoll, 1914). Procain hydrochloride administered to the excised eye results in a reduction in magnitude of phase 2, and little or no change in magnitude of phase 1.

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STUDIES ON THE ANAEROBIC METABOLISM AND THE AEROBIC CARBOHYDRATE CONSUMPTION OF SOME FRESH WATER SNAILS

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Field work attempting the eradication of schistosome-transmitting snails in various parts of the world has demonstrated the inadequacy of the hitherto available molluscacides. If control of schistosomiasis is to be attempted by means of chemical compounds interfering with the metabolic pathways of the intermediate hosts of the flukes, the establishment of a theoretical basis for further evaluation of potential and actual molluscacides is urgently needed.

In a previous paper by von Brand, Nolan, and Mann (1948), some data on the aerobic faculties of various species of snails are recorded. A detailed knowledge of the anaerobic metabolism of such snails is also necessary since, according to a personal communication by Dr. W. H. Wright, some schistosome-transmitting snails have the ability to escape the action of poisons by burrowing into the mud, which is usually very poor in oxygen.

In the present paper an attempt is made to answer some unsolved problems concerning the anaerobic metabolism of snails which, in fact, has so far never been studied. Specifically, the following points have been investigated: Anaerobic resistance of various species, the anaerobic carbohydrate consumption, carbon dioxide and lactic acid production, and the quantitative relationships between anaerobic and aerobic carbohydrate consumption.

Material and Methods

The following species of snails were employed and where no further information is provided they were of the same derivation as those used in a previous study (von Brand, Nolan, and Mann, 1948).

1. Pulmonates

PLANORBIDAE: *Australorbis glabratus; Helisoma duryi; Helisoma trivolvis* specimens collected near Brownsville, Texas; *Tropicorbis obstructus; Tropicorbis donbilli; Planorbarius corneus,* laboratory-reared; *Biomphalaria boissyi,* laboratory-reared from Egyptian specimens; *Biomphalaria pfeifferi,* laboratory-reared from Liberian stock.

LYMNAEIDAE: *Lymnaea stagnalis* and *Lymnaea palustris,* both laboratory-reared from Douglas Lake, Michigan stock; *Lymnaea natalensis,* laboratory-reared from Dharan, Saudi Arabia, specimens.

[1] The authors wish to express their appreciation for the contribution of snails to Dr. E. G. Berry, Mrs. M. O. Nolan, Dr. L. Olivier, and Mr. W. B. DeWitt of this Laboratory and Dr. H. van der Schalie and Mr. E. Abdel-Malek of the University of Michigan.

PHYSIDAE: *Physa gyrina; Physa cubensis,* laboratory-reared from Cuban stock; *Aplexa nitens,* laboratory-reared from specimens collected near Brownsville, Texas.

2. Operculates

AMNICOLIDAE: *Oncomelania nosophora,* laboratory-reared from Japanese stock.

POMATIOPSIDAE: *Pomatiopsis lapidaria.*

THIARIDAE: *Melanoides tuberculatus,* laboratory-reared from Dharan, Saudi Arabia, specimens.

PLEUROCERIDAE: *Goniobasis livescens,* used shortly after being shipped from northern Michigan.

All determinations were carried out at 30° C., a temperature corresponding approximately to the summer temperature in the Washington, D. C., area. It was well tolerated by all snails.

Warburg equipment was used throughout the work, flasks of about 17 and 5 ml. being employed for larger and smaller snails respectively. Two ml. of dechlorinated tapwater served as medium in the former, one ml. in the latter. Anaerobic conditions were established by passing a stream of 99.99 per cent pure Linde nitrogen, further purified by passing over heated copper, through the shaking flasks for 15 to 20 minutes.

In the aerobic experiments the previously described technique was again employed (von Brand, Nolan, and Mann, 1948).

The total carbohydrates were determined in the snail tissues by Dische and Popper's (1926) method, the color being read in a Fisher electrophotometer with a filter at 425 mμ, instead of in a visual colorimeter.

Barker and Summerson's (1941) method was used for the lactic acid determinations in the snail tissues and the medium.

RESULTS

1. *Anaerobic resistance*

Considerable differences in anaerobic resistance between various groups of snails occurred. The figures summarized in Table I show that the Lymnaeidae and Physidae withstood anaerobic conditions without apparent damage for only 6 hours, and that most died within 16 hours. The Planorbidae and the operculate snails were more resistant, most species surviving regularly for at least 24 hours, and some, especially *Helisoma trivolvis* and *Melanoides tuberculatus,* for 48 to 64 hours. *Australorbis glabratus* and *Biomphalaria pfeifferi,* however, were somewhat more sensitive, all specimens surviving regularly only 16 hours in the lack of oxygen.

The behavior of the snails under anaerobic conditions was quite characteristic. All extended maximally out of their shells and soon, at the latest within a few hours, became completely motionless. If not used for chemical determinations, the snails, after the end of the anoxic period, were placed into beakers containing aerated dechlorinated tapwater. As long as they were fully extended, they recovered completely, resuming motion soon after restoration of aerobic conditions. If the anaerobic period lasted too long, on the contrary, the snails began to hemorrhage and finally retracted into their shells. This seemingly indicates that the above-

mentioned lack of motion was not a complete paralysis. Snails which had retracted into their shells during the anaerobic period did not recover during a subsequent aerobic period. Whether, in all cases, they actually died during the anaerobic period, or died shortly thereafter, could not be determined.

2. *Anaerobic metabolism*

A condensed summary of our experimental data (Table II) shows that, in general, two series were carried out with a given species of snails. In one series the CO_2 production and carbohydrate consumption were studied. In the second series the CO_2 production, the lactic acid content of the tissues, and the excretion of lactic acid into the medium were determined. The carbon dioxide determination then was common to both series. A survey of the figures shows that in pulmonates the

TABLE I

Anaerobic resistance of various species of fresh water snails at 30° C.

Species	Period of anaerobiosis									
	6 hours		16 hours		24 hours		48 hours		64 hours	
	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving	Number of specimens	Per cent surviving
Australorbis glabratus	72	100	59	98	12	25	12	0		
Helisoma duryi					36	100				
Helisoma trivolvis			6	100	6	100	6	100	5	100
Tropicorbis obstructus					110	100			12	25
Tropicorbis donbilli	12	100			12	66	6	0		
Planorbarius corneus	36	100			42	100			12	17
Biomphalaria boissyi	12	100	36	100	12	100	12	0		
Biomphalaria pfeifferi			67	100	12	50	12	0		
Lymnaea stagnalis	36	100	12	9						
Lymnaea palustris	36	100	12	0						
Lymnaea natalensis	48	100	12	0						
Physa gyrina	64	100	33	3						
Physa cubensis	72	100	6	0						
Aplexa nitens	36	97	12	0						
Oncomelania nosophora					30	100	18	17		
Pomatiopsis lapidaria					108	98	12	25		
Melanoides tuberculatus					60	100	12	92	12	84
Goniobasis livescens					12	100				

CO_2 figures of both series were in most cases in reasonably close agreement, while considerably greater differences occurred in this respect in the two operculates *Pomatiopsis lapidaria* and *Melanoides tuberculatus*, so tested. No definite reason for the irregular behavior of these latter can be given.

The length of the anaerobic period was chosen for each species in accordance with the resistance data discussed in the preceding section. In the cases of *Australorbis glabratus* and *Planorbarius corneus*, two periods of different duration were tested in order to see if significant differences in metabolism occurred. The shorter period corresponded to that used with the nonresistant species. The data (Tables II and III) gained from both periods agreed fairly well, with the exception of an unexplained larger excretion of lactic acid by Planorbarius during the shorter period. There is, therefore, no reason to assume that the differences in metabolism between

resistant and nonresistant species mentioned below are correlated with the different lengths of anaerobic periods employed.

From the data summarized in Table II, the average hourly rate of metabolic processes has been calculated (Table III), and a perusal of both Tables II and III reveals the following facts.

Total carbohydrates. The highest carbohydrate content, 3.5 per cent of the fresh weight, was found in *Lymnaea natalensis,* while *Physa gyrina* showed the lowest, 1 per cent. The rates of anaerobic carbohydrate consumption of well-fed snails were highest in the Physidae and Lymnaeidae, intermediate in most Planorbidae, and lowest in operculates. The nutritional state of the snails was of consid-

TABLE II

Anaerobic metabolism of various species of snails

Species	No. of exper.[1]	Anaerobiosis	Entire exper. period. Carbon dioxide mm.[3] per 1 gm.[1]	Carbohydrates in per cent		Lactic acid in tissues microgram per gm.		Lactic acid excreted microgram per gm.
		Hours		Initial	Final	Initial	Final	
Australorbis glabratus[2]	24; 24	16	641 ±20; 664 ±26	2.69 ±0.18	2.19 ±0.15	232 ±21	155 ± 29	422 ±121
Australorbis glabratus[3]	36; 36	6	187 ± 9; 203 ±14	2.58 ±0.19	2.47 ±0.15	150 ±22	150 ± 18	118 ± 25
Australorbis glabratus[3]	18	16	327 ±22	1.35 ±0.06	1.11 ±0.12			
Australorbis glabratus[4]	22	16	219 ±27	0.54 ±0.12	0.32 ±0.05			
Helisoma duryi	18; 18	24	819 ±36; 528 ±35	2.08 ±0.18	1.00 ±0.13	106 ±15	104 ± 19	114 ± 36
Tropicorbis obstructus	17; 18	24	1177 ±43; 1024 ±56	1.77 ±0.13	0.67 ±0.07	160 ±34	148 ± 21	357 ± 37
Planorbarius corneus	24; 17	24	760 ±44; 709 ±33	2.94 ±0.02	2.65 ±0.18	267 ±66	202 ± 38	131 ± 32
Planorbarius corneus	18; 24	6	141 ±12; 143 ±12	2.35 ±0.34	2.27 ±0.29	285 ±12	205 ± 11	252 ± 14
Biomphalaria boissyi	18; 17	16	646 ±35; 637 ±23	2.39 ±0.17	1.70 ±0.13	320 ±20	265 ± 24	692 ± 60
Biomphalaria pfeifferi	18; 18	16	752 ±45; 637 ±37	2.92 ±0.19	2.07 ±0.23	229 ±23	207 ± 32	506 ± 86
Lymnaea stagnalis	18; 18	6	479 ±34; 567 ±61	1.26 ±0.10	0.99 ±0.11	247 ±47	689 ±102	871 ±109
Lymnaea palustris	18; 18	6	300 ±34; 395 ±46	1.22 ±0.13	0.71 ±0.07	112 ±16	664 ± 87	34 ± 5
Lymnaea natalensis	18; 18	6	284 ±20; 237 ±24	3.55 ±0.17	3.25 ±0.10	302 ±48	756 ± 97	1000 ± 77
Physa gyrina	18; 17	6	144 ±11; 214 ±23	0.98 ±0.07	0.49 ±0.06	85 ± 9	313 ± 41	45 ± 9
Physa cubensis	17; 18	6	313 ±35; 395 ±46	1.22 ±0.11	0.76 ±0.08	185 ±34	590 ±161	63 ± 8
Aplexa nitens	17; 18	6	277 ±19; 371 ±21	2.05 ±0.10	0.94 ±0.11	185 ±17	384 ± 35	679 ± 66
Oncomelania nosophora	18	24	316 ±35	1.42 ±0.12	0.99 ±0.08			
Pomatiopsis lapidaria	12; 16	24	290 ±42; 646 ±89	1.47 ±0.08	1.05 ±0.08	114 ±25	98 ± 25	188 ± 45
Melanoides tuberculatus	18; 29	24	322 ±35; 177 ±24	2.73 ±0.13	2.37 ±0.13	89 ±20	128 ± 24	120 ± 34
Goniobasis livescens	12	24				97 ±13	93 ± 7	191 ± 24

[1] The first figures in these columns refer to the carbohydrate series; the second figures to the lactic acid series.

[2] Well-fed snails.

[3] Snails from an aquarium with scanty food supply.

[4] Snails starved for 2 weeks prior to the determinations.

The figures behind the plus and minus signs represent the standard error of the mean.

erable influence as is seen by the example of *Australorbis glabratus* which, after 2 weeks' starvation, consumed less than half the amount of endogenous carbohydrate catabolized by well-fed specimens.

Lactic acid. The lactic acid content of the tissues of snails taken from aerated aquaria was relatively low, varying from about 9 mg. per cent in *Physa gyrina* or *Melanoides tuberculatus* to about 30 mg. per cent in *Lymnaea natalensis.* In contrast to these relatively small variations in preanaerobic lactic acid level, a very distinct difference in the anaerobic lactic acid levels was observed in different species. The Lymnaeidae and Physidae showed a marked accumulation of lactic acid within their tissues, while this did not occur in the Planorbidae and the operculate snails. In most species belonging to these latter groups the anaerobic level was even lower

than the aerobic one. While most of these latter differences do not appear statistically significant, their repeated occurrence suggests a lactic acid excretion during anaerobiosis in excess of the amounts formed.

All species studied excreted lactic acid. The total production of lactic acid during the anaerobic period (lactic acid excreted plus lactic acid accumulated in the tissues, or lactic acid excreted minus lactic acid deficit in the tissues) was largest in Lymnaeidae and Physidae, but small in the other species.

TABLE III

Hourly anaerobic metabolic changes of various species of snails per 1 gm. tissue

Species	CO_2 mm.[3] produced	Carbohydrate consumed, mg.	Lactic acid accumulated in tissues, mg.	Total lactic acid produced, mg.	Carbon balance per cent of total CO_2 liberated from bicarbonate by lactic acid	Per cent of consumed carbohydrate carbon accounted for by lactic acid
Australorbis glabratus[1]	41	0.31	0	0.022	13	7
Australorbis glabratus[2]	33	0.18	0	0.020	15	11
Australorbis glabratus[3]	20	0.15				
Australorbis glabratus[4]	14	0.14				
Helisoma duryi	28	0.45	0	0.005	4	1
Tropicorbis obstructus	46	0.46	0	0.014	8	3
Planorbarius corneus[5]	30	0.12	0	0.003	3	3
Planorbarius corneus[6]	24	0.13	0	0.029	29	22
Biomphalaria boissyi	40	0.56	0	0.040	25	7
Biomphalaria pfeifferi	43	0.53	0	0.030	17	6
Lymnaea stagnalis	87	0.45	0.074	0.219	63	49
Lymnaea palustris	58	0.85	0.092	0.098	41	12
Lymnaea natalensis	43	0.50	0.076	0.243	139	50
Physa gyrina	30	0.82	0.038	0.046	38	5
Physa cubensis	59	0.77	0.068	0.078	32	10
Aplexa nitens	54	1.85	0.033	0.146	67	8
Oncomelania nosophora	13	0.18				
Pomatiopsis lapidaria	20	0.18	0	0.007	9	4
Melanoides tuberculatus	10	0.15	0.002	0.007	17	5
Goniobasis livescens	9		0	0.008		

[1] Well-fed snails, 16 hours anaerobiosis.
[2] Well-fed snails, 6 hours anaerobiosis.
[3] Snails from an aquarium with scanty food supply.
[4] Snails starved for 2 weeks prior to the determinations.
[5] Well-fed snails, 24 hours anaerobiosis.
[6] Well-fed snails, 6 hours anaerobiosis.

The last column of Table III shows conclusively that lactic acid is, from a quantitative standpoint, a major end product of the anaerobic carbohydrate metabolism only in *Lymnaea stagnalis* and *Lymnaea natalensis*. In all other species, unidentified end products must predominate by far.

Carbon dioxide. The carbon dioxide excretion showed fairly large variations from species to species. Due to the calcareous shells it was, unfortunately, impossible to study the question of carbon dioxide retention in the tissues. Although the CO_2 figures are probably not absolutely correct, it is believed that the following conclusions are not far from the truth.

The next to the last column of Table III shows that in Lymnaeidae and Physidae a major portion of the carbon dioxide excreted was probably of inorganic origin, having been liberated from bicarbonate during glycolysis leading to lactic acid. In *Lymnaea natalensis* even more lactic acid was found than corresponded to the carbon dioxide excreted. In all other species the amount of CO_2 due to lactic acid was only moderate. In view of the absence of information concerning the other end products of the anaerobic carbohydrate metabolism, the question of whether or not the remaining CO_2 was also of inorganic origin or whether it represented true respiratory CO_2 must remain unanswered at the present time.

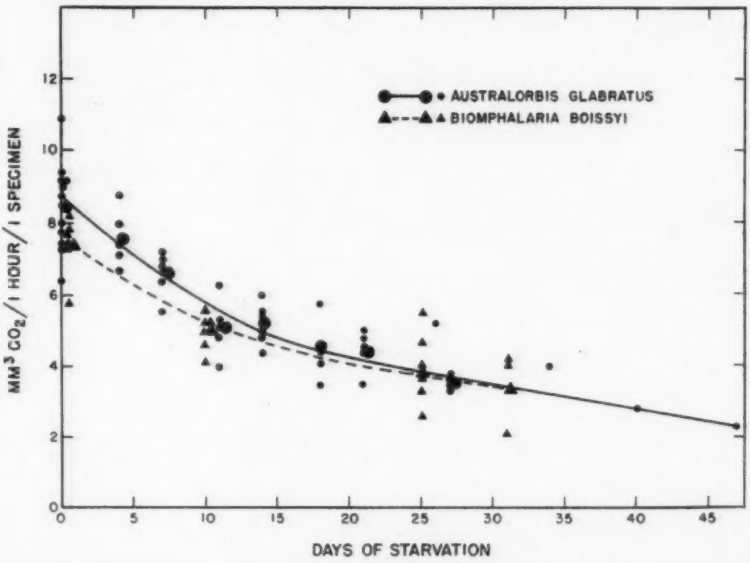

FIGURE 1. Influence of starvation on the anaerobic carbon dioxide production of aquatic pulmonate snails. Twelve well-fed specimens of *Australorbis glabratus* and 6 specimens of *Biomphalaria boissyi* were used. They were exposed on specified days to 6 hours anaerobiosis. Between the anaerobiosis periods the snails were kept starving in aerated, dechlorinated tapwater. The determinations were continued until the last snail of each group died. The figure shows the individual and the mean values (small and large symbols respectively).

During starvation (Fig. 1) the rate of anaerobic CO_2 production fell progressively but the decline was, on the whole, less pronounced than in the case of the previously studied aerobic metabolism (von Brand, Nolan, and Mann, 1948).

In several species the specimens used for the CO_2 determinations varied rather markedly in size. A study of this material (Fig. 2) shows that in intraspecific comparisons the CO_2 production followed the surface law rather well (relative surface calculated as weight $^2/_3$), while the same obviously did not hold true for all cases of interspecific comparison.

3. *Aerobic carbohydrate metabolism*

The data summarized in Table IV demonstrate that all species consumed carbohydrate under aerobic conditions. The aerobic rate was always smaller than the anaerobic one as indicated by the ratio between anaerobic and aerobic rate given in the last column of Table IV. It is noteworthy, however, that the quotient was small in 5 out of the 7 species tested.

In *Planorbarius corneus* and *Pomatiopsis lapidaria* the amount of oxygen consumed by the snails was clearly in excess of the amount required for complete oxidation of the consumed carbohydrate. In the other cases it was either just

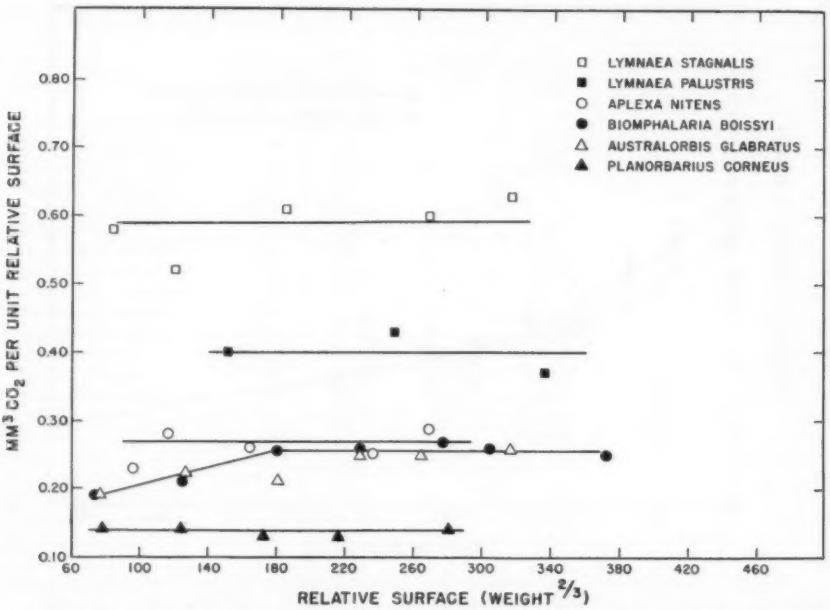

FIGURE 2. Relation between anaerobic carbon dioxide production and relative surface of pulmonate aquatic snails. The values are mean values of groups of snails varying in number between 3 and 20.

sufficient to account theoretically for complete oxidation, or insufficient. It must be assumed that in these latter cases some incomplete oxidations occurred. This is probably also true for *Australorbis glabratus* and *Helisoma duryi* since it is unlikely that they metabolize carbohydrate exclusively. The previously reported data concerning the respiratory quotient of *Australorbis glabratus* (von Brand, Nolan, and Mann, 1948) exclude an assumption of exclusive carbohydrate utilization, at least for this species.

The lactic acid level of the snail tissues remained practically unchanged in most species under aerobic conditions. In *Australorbis glabratus,* however, a rather

TABLE IV

Aerobic carbohydrate metabolism of some snail species

Species	Number of experiments	Experimental period, hours	Oxygen consumed mm³. per 1 gm.		Carbohydrates			Lactic acid in tissues microgram per 1 gm.		Oxygen required for total oxidation of consumed carbohydrates in per cent of oxygen consumed	Ratio $\frac{\text{anaerobic}}{\text{aerobic}}$ carbohydrate consumption
					Per cent in tissues		Mg. consumed per 1 gm. in 1 hour				
			Total period	1 hour	Initial	Final		Initial	Final		
Australorbis glabratus	18	16	2544 ±136	159	2.69 ±0.18	2.35 ±0.14	0.21	232 ±21	65 ±22	99	1.5
Helisoma duryi	29	24	1937 ±105	81	2.08 ±0.18	1.81 ±0.29	0.11	133 ±26	114 ± 9	104	4.1
Planorbarius corneus	18	24	2870 ±165	120	2.94 ±0.02	2.89 ±0.28	0.02	267 ±66	248 ±41	13	2.4
Lymnaea stagnalis	18	6	1230 ± 89	205	1.26 ±0.10	1.04 ±0.10	0.37	247 ±47	179 ±42	133	1.2
Lymnaea palustris	18	6	821 ±193	137	1.22 ±0.13	1.00 ±0.12	0.37	112 ±16	103 ±14	200	2.3
Pomatiopsis lapidaria	18	24	2230 ±108	93	1.47 ±0.08	1.37 ±0.08				30	4.5
Melanoides tuberculatus	18	24	1285 ± 90	54	2.73 ±0.13	2.46 ±0.16	0.11	89 ±20	98 ±33	156	1.4

sharp decline in lactic acid level occurred during the period of observation. No reason for this aberrant behavior can be given at the present time.

DISCUSSION

The fact that aquatic snails possess a certain tolerance towards the lack of oxygen has been known for some time. Alsterberg (1930) found the operculates Bulinus sp. and Vivipara sp. more resistant than the pulmonates *Lymnaea ovata, Lymnaea stagnalis,* and *Lymnaea truncatula. Planorbarius corneus* was only slightly more resistant than the lymnaeids. On the other hand, Raffy and Fischer (1933) reported Planorbarius was much more resistant than Lymnaea upon total immersion into oxygen-poor water. The present results, carried out under completely anaerobic conditions, are more in agreement with the latter than the former. In our experiments the Lymnaeidae and Physidae were considerably less resistant than the Planorbidae or the different operculates tested.

The anaerobic metabolic level of the nonresistant species, as expressed by the rates of CO_2 production and carbohydrate consumption, was, in general, somewhat higher than that of the resistant species, but a certain overlapping occurred. It is dubious, therefore, whether the metabolic level as such has a decisive bearing on the question of what factors are responsible for the differential sensitivity towards lack of oxygen. The metabolic level of snails depends, at a given temperature, on the nutritional state. Whether this last factor materially influences the length of anaerobic survival remains to be investigated.

A well defined difference between resistant and nonresistant species was found in respect to lactic acid. No accumulation whatever occurred during anaerobic periods in the tissues of the former; they excreted all the new-formed and possibly even some of the preformed lactic acid. On the contrary, the lactic acid level in the tissues of the nonresistant species increased rather sharply; these species were

capable of excreting only part of the newly formed acid. How far this accumulation of lactic acid within the tissues was responsible for the early death of the animals cannot be decided at the present time. It is hardly likely that it was the only factor involved. It must be remembered in this connection that those species which do not show such an accumulation of lactic acid ultimately die of asphyxiation.

All species consumed carbohydrate anaerobically at a somewhat faster rate than under aerobic conditions, but only in *Helisoma duryi* and *Pomatiopsis lapidaria* was the quotient $\frac{\text{anaerobic consumption}}{\text{aerobic consumption}}$ as large as that in most hitherto-studied free-living invertebrates (older literature in von Brand, 1946; newer data in Cleary, 1948). In the other species the quotient was small, in *Australorbis glabratus*, *Lymnaea stagnalis*, and *Melanoides tuberculatus* as small, indeed, as in parasitic worms. It is difficult to adduce definite reasons for this rather curious observation. A low carbohydrate consumption quotient may result under anaerobic conditions, if the snail survives by producing a relatively small amount of energy; then the anaerobic carbohydrate consumption may proceed at a relatively low rate even in comparison with the much more efficient oxidative breakdown. It seems very probable that such a reduction in energy production is involved, at least to some degree, in the present case. It is quite certain that these snails possess only a tolerance towards anaerobiosis, but are not capable of leading an anaerobic life in the true sense of the word.

However, a second factor may also be involved. One of the reasons why the above carbohydrate quotient is low in many parasitic worms is the fact that they have a metabolism characterized by aerobic fermentations, which are less efficient in energy production than completely aerobic respiration and therefore increase the aerobic carbohydrate consumption, thus lowering the quotient. Our findings concerning the quantitative relationships between oxygen and carbohydrate consumption seem to indicate that such a situation may also prevail in some snails. This point will have to be clarified by further work.

It has been previously pointed out (von Brand, Nolan, and Mann, 1948) that the aerobic metabolic levels of pulmonate and operculate snails are different, but that the oxygen consumption follows the surface law both in intraspecific and interspecific comparisons. The present study reveals that, at least, in pulmonate snails (no suitable material of operculates was available), the anaerobic carbon dioxide production follows the surface law in intraspecific comparison. This seems to be the first instance that such a relationship has been established for the anaerobic metabolism of invertebrates. The same relationship, however, definitely does not hold in all cases of interspecific comparison. The reasons for differences in anaerobic carbon dioxide excretion by various species of snails may be different. Our observations on the behavior of lactic acid revealed considerable differences between species. In Lymnaeidae and Physidae this acid accounted for a rather large percentage of the excreted carbon dioxide, in other species only for a small one. It is then quite possible that different metabolic processes lead to different rates of carbon dioxide production depending upon the proportions of inorganically and organically derived carbon dioxide. Similar considerations apply to the rates of anaerobic carbohydrate consumption, various fermentative pathways liberating different amounts of energy. Whether or not fixed relationships exist between rates of anaerobic

metabolism and size of snails belonging to different species, can probably be determined only after the actual energy production of the various processes has been elucidated.

A rather interesting result of our experiments is the observation that lactic acid, although produced by all snails, accounts in several species only for a small fraction of the anaerobically consumed carbohydrate; while in others it obviously represents a major end product. Snails had never before been studied in this respect, but similar differences are well known to occur in parasitic worms (review of the literature in Bueding, 1949), protozoa (literature in von Brand, 1950), as well as mollusks other than snails. Dugal and Fortier (1941) found no anaerobic lactic acid production in oysters, nor did Wernstedt (1944) in Dreissensia; while the production of rather large amounts of lactic acid have been reported in the case of Venus (Dugal, 1939). Recently, however, Humphrey (1949) observed some lactic acid production also in oysters.

Our studies indicate that the consumption of carbohydrate is probably of major importance in allowing snails to survive the adverse condition of lack of oxygen. It would then seem justifiable to attempt the destruction of snails that are present in anaerobic or semi-anaerobic habitats, by the application to such habitats of chemical compounds known to interfere with the glycolytic enzymes.

Summary

1. Lymnaeidae and Physidae tolerated complete lack of oxygen less well than Planorbidae or operculates belonging to different families.

2. All species consumed carbohydrate under anaerobic conditions and produced carbon dioxide and lactic acid. While in several species the lactic acid produced was sufficient to account for all or a large part of the carbon dioxide as liberated from bicarbonate, this was not the case in other species.

3. The anaerobic metabolic level as measured by carbon dioxide production and carbohydrate consumption of the resistant species was, on an average, lower than that of the nonresistant ones. The former did not accumulate lactic acid within their tissues during an anaerobic period, while the latter did so to a marked degree.

4. In most species the anaerobic carbohydrate consumption was only slightly higher than the aerobic rate. One of the reasons for this may be the probable occurrence of aerobic fermentations in these species.

5. Lactic acid was quantitatively a major end product of the anaerobic carbohydrate consumption only in *Lymnaea stagnalis* and *Lymnaea natalensis;* in all other species unidentified end products must have prevailed.

6. The anaerobic carbon dioxide production followed the surface law in intraspecific comparisons in pulmonates rather closely, but definitely not in all cases of interspecific comparisons.

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AN ANALYSIS OF THE EFFECTS OF A SACCULINID PARASITE ON THE EXTERNAL MORPHOLOGY OF CALLINECTES SAPIDUS RATHBUN

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INTRODUCTION

There is no decapod crustacean that has been more intensively studied by American zoologists than the common and commercially valuable blue crab, *Callinectes sapidus* Rathbun. Accordingly, when we find such a well-known species infested with a rhizocephalan parasite an analysis of the effects of this type of parasitism is greatly facilitated through knowledge already available regarding the normal crab. On the other hand, it also happens that an understanding of the abnormal may shed additional light on the normal. The problem, therefore, of the relation of the parasite *Loxothylacus texanus* Boschma to the blue crab has been undertaken primarily to increase knowledge of the nature and effects of sacculinization, but also with the hope of contributing, if possible, to a better understanding of the normal host organism itself.

Much has been written concerning host-parasite relationships in the Sacculinidae and the general problem of "parasitic castration." Giard (1886, 1887), who coined the latter term, was the first to point out that male crabs infested with Sacculina often develop in a female direction as shown by changes in the form of the abdomen, the claws, and the pleopods, while the testes become more or less atrophied. Parasitized females, on the other hand, show little or no external changes. Other important observations on external modifications produced by sacculinids on their hosts were reported by Smith (1906, 1910), Potts (1910), Pérez (1933), Day (1935), Okada and Miyashita (1935), Bulgurkov (1938), and Veillet (1945). In general, it may be said that various species of sacculinids differ with respect to the extent of modifications they induce in their hosts, and variations also appear when one compares different species of hosts infested by the same parasite. A fairly complete bibliography of the literature on parasitic castration may be found in Hanström (1939) and Brown (1944).

The parasite reported on here is structurally similar to Sacculina; in fact, Boschma (1928) established the genus Loxothylacus for certain species of Rhizocephala that were included up to that time in the genus Sacculina. *Loxothylacus texanus*, described by Boschma in 1933, has been reported only from the region of the Gulf of Mexico where it parasitizes *Callinectes marginatus* (A. Milne-Edwards) as well as *C. sapidus* Rathbun.

[1] The author is happy to express his appreciation to Sister Mary Gerald Shea who collaborated in the preliminary phases of the work. Aid from The Catholic University of America Research Fund is also acknowledged. Most of the specimens of parasitized crabs used in this study were secured through the courtesy of the Game, Fish and Oyster Commission, Rockport, Texas, and of Mr. J. W. Hedgpeth, to whom thanks are also due.

Materials Examined

Thirty parasitized crabs of the species *C. sapidus* were used in this study, with both sexes almost equally represented. The parasite is found attached to the underside of the abdomen of the host (Fig. 1), with the stalk of attachment fastened to the midline of the fourth or fifth abdominal segment, rarely on the third. As a rule, one parasite occurs on a single host, but instances of multiple infestation are not uncommon. Of 30 infested Callinectes, 18 bore one parasite, 7 had 2 parasites, 3 were triply infested, and 2 had 4 parasites apiece. The locality and collection data are as follows:

Figure 1. The sacculinid *Loxothylacus texanus* attached to the underside of the abdomen of *Callinectes sapidus*. The crab is an immature female of 52 mm. carapace width, but in the shape of the abdomen it resembles a normal adult female.

Florida: Cedar Keys, Oct.–Nov., 1947, 5 specimens on 2 crabs, E. Lowe Pierce coll. U.S.N.M. 89223.

Texas: Galveston Bay, April 16, 1938, 3 specimens on 2 crabs, W. G. Hewatt coll. U.S.N.M. 80465. Cedar Bayou, Nov. 14, 1947, 6 specimens on 4 crabs, Joel W. Hedgpeth coll. Aransas Bay, April, 1942, 4 specimens on 2 crabs, Gordon Gunter coll. Aransas Bay, May–July, 1948, 31 specimens on 20 crabs, Game, Fish and Oyster Commission, Rockport, Texas.

Linear measurements of the carapaces of the crabs were made according to the method presented by Gray and Newcombe (1938). Using the width as the basic index, the sizes ranged in millimeters from 38 × 19 to 75 × 35 for males, and from 40 × 18 to 85 × 38 for females. About forty normal specimens of Callinectes,

selected to give the same size range as the parasitized crabs, were studied for purposes of comparison.

Effect of the Parasite on the Host

The amount of change effected by a rhizocephalan parasite in the secondary sex characters of the host appears to be correlated with the degree of differences existing between the normal males and females. Where the differences between the sexes are small, the amount of modification is also slight. *Callinectes sapidus* displays a high degree of sexual dimorphism and, correspondingly, the changes resulting from parasitism are very great. Normal male and female blue crabs are easily distinguished by the shape of the abdomen and the nature of the abdominal appendages. The adult female abdomen is broad and semicircular in outline and is composed of six distinct segments. The male abdomen has a narrow, "T"-shaped appearance, with only four of the six segments distinct, due to the fact that segments 3, 4, and 5 have fused. The pleopods of the female are biramous appendages with the exopodite slightly shorter than the endopodite. They occur on the second, third, fourth and fifth abdominal segments and the endopodite particularly is furnished with long hairs to which the eggs are attached in ovigerous specimens. The abdominal appendages of the male consist of two pairs of copulatory styles, the second being inserted into the first for the purpose of sperm transfer. Juvenile specimens are distinguishable as to sex on the same basis as the adults except that the abdomen of the immature female lacks the distinctive breadth of the adult and is triangular in shape. Further details regarding the external differences between the sexes may be found in the papers of Churchill (1919), Cochran (1935), Truitt (1939), Cronin (1947), and others.

Loxothylacus texanus evidently attacks the smaller crabs that have not yet reached maturity. The parasitized crabs varied in size from 38 mm. to 85 mm. carapace width, which is considerably below the minimal size (140 mm. carapace width) given for sexually mature Callinectes by Gray and Newcombe (1938) and Cronin (1947).

A histological study of the gonads of the parasitized crabs was not considered worth while because of the immaturity of the specimens and the absence of controls from the same locality. However, dissection of a representative sample of normal and infested crabs of comparable carapace widths clearly indicated that the gonads of the parasitized specimens were of much smaller size.

Modification of the Male Host

The most pronounced change in the infested male is the broadening of the abdomen to such a degree that it closely resembles that of a normal female (Fig. 2). This type of modification is perhaps the one most commonly noted by previous writers on the effects of sacculinization on Brachyuran crabs (Giard, 1887; Smith, 1906; Potts, 1910; Pérez, 1933; Day, 1935; Okada and Miyashita, 1935; Bulgurkov, 1938; Veillet, 1945). Measurements of the extent of modification based upon a comparison between the width of the carapace and the width of the abdomen yielded no evidence that either the size of the parasite or the number of parasites per host was related to amount of deviation from the normal. Moreover, there

seemed to be no relationship evident between the degree of modification and the actual size of the host.

Broadening of the parasitized male abdomen is accompanied by an increase in the number of hairs around the margins. In normal male Callinectes the marginal hairs are more or less confined to the first four segments of the abdomen, but in the majority of parasitized specimens they are, as in the normal female, present on all the segments, forming an uninterrupted fringe. Pérez (1933) likewise noted a feminine type of villosity in sacculinized male Pachygrapsus.

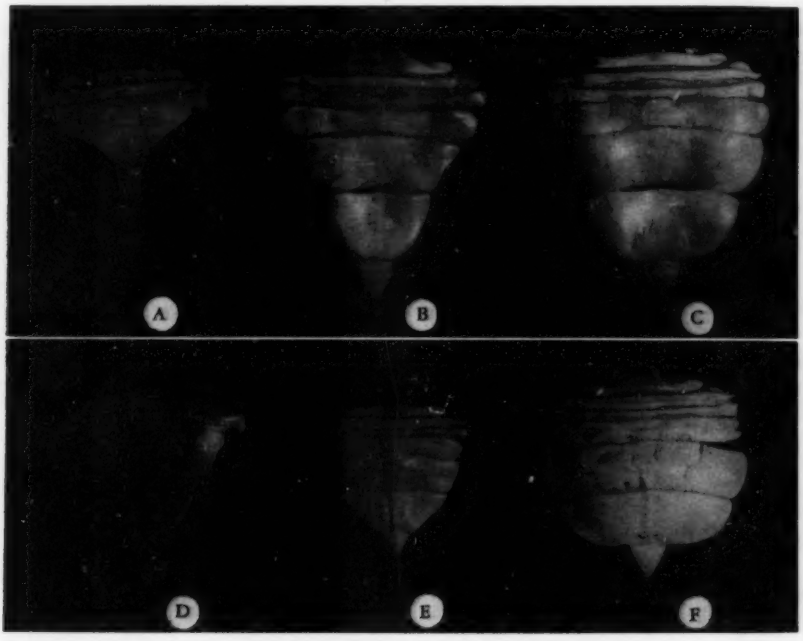

FIGURE 2. Abdomens of normal and parasitized *Callinectes sapidus*.
A. Normal male. B, C. Males parasitized by Loxothylacus. D. Normal immature female. E, F. Immature females parasitized by Loxothylacus.

Parasitism of the male also brings about complete segmentation of the abdomen. The third, fourth and fifth abdominal segments, previously coalesced, now become distinct and freely movable. Giard (1887) made a similar observation regarding *Carcinus moenas* and *Portunus holsatus* infested by sacculinids, and Potts (1910) and Veillet (1945) confirmed this in Carcinus. Day (1935) restudied the case of Portunus and showed that redivision of the male abdominal segments varied in degree, with the maximum extent more liable to occur in the smaller crabs.

The difference in abdominal segmentation between the sexes in Callinectes, as Cochran (1935) has shown, is also associated with a difference in dorsal muscula-

ture. In the female each abdominal somite has a pair of dorsal muscles, but in the male the fused section has only two pairs of dorsal muscles.

Dissection of the parasitized males, to discover whether or not total segmentation of the abdomen affects the dorsal musculature, revealed that each abdominal segment was equipped with a pair of dorsal muscles exactly as in the female (Fig. 3). Evidently a new pair of muscles arises in the previously fused region so that the three segments into which it becomes divided are each furnished with a separate pair of dorsal muscles. We are of the opinion that it is the muscles of the third somite which arise *de novo*, because the area which becomes the third somite is originally without trace of dorsal muscles, while the two pairs of existing muscles lie in the center and at the posterior region respectively of the fused section.

Turning now to the appendages of the male abdomen we find further evidence of feminization in the case of the second pleopod. This appendage is constructed on

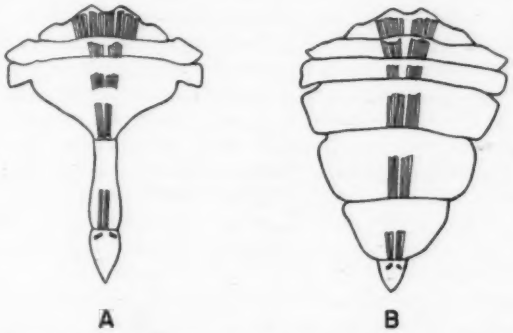

FIGURE 3. Effects of sacculinization on the segmentation and musculature of the male abdomen.

A. Abdomen of normal male Callinectes showing dorsal muscles and fusion of segments 3, 4 and 5. B. Abdomen of male parasitized by Loxothylacus. Its musculature and segmentation are like those of a normal female.

the same basic plan as the first pleopod but is much smaller and weaker. It consists of three parts (Fig. 4E): a stout, flat coxopodite; a small basipodite; and a thin end-piece usually spoken of as the "flagellum." This terminal prong is normally kept inserted in the posterior foramen of the first pleopod since its function is to force spermatophores through the much longer tube-like "flagellum" of the first pleopod which is the primary intromittent organ.

In parasitized specimens the second pleopod was never found inserted into the first and in every case its structure showed varying degrees of modification. A slightly modified type (Fig. 4F) is characterized by a shortening of the coxopodite and the bending of the "flagellum" away from the protopod so that the appendage changes from V-shaped to L-shaped in appearance. Further modification results in the condition shown in Figure 4G. Here the coxopodite has grown still smaller but the basipodite has enlarged. The terminal "flagellum" has turned still farther to the rear and its surface, previously smooth, is now furnished with short hairs.

Two of the parasitized males showed the extreme modification pictured in Figure 4I. Here the pleopod has become biramous and possesses a well-developed expodite complete even to the presence of long plumose hairs. The "flagellum" remains as the endopodite, and the entire appendage, while not identical in appear-

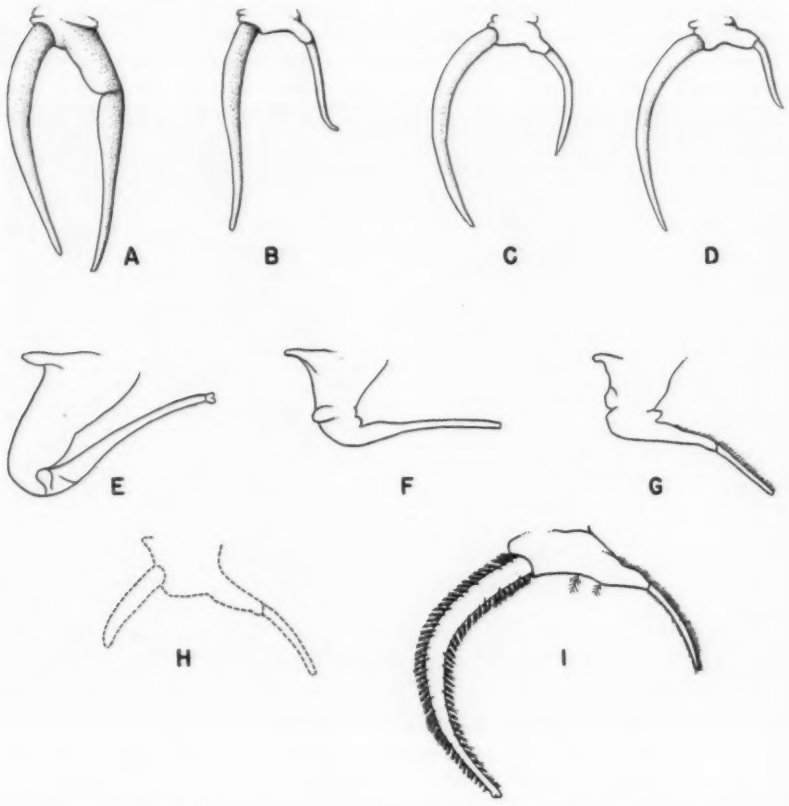

FIGURE 4. Pleopods of normal and parasitized specimens of *Callinectes sapidus*.
A. Normal female. B, C, D. Examples of female pleopods showing effects of parasitism by Loxothylacus. E. Second abdominal appendage of normal male. F, G. Minor modifications of second male pleopod resulting from parasitism. H. Hypothetical stage in transformation of male pleopod into a female type of appendage. I. Second pleopod of parasitized male showing extreme modification. This type of appendage is indistinguishable from that of a parasitized female.

ance with that of a normal female, is certainly indistinguishable from the pleopod of a parasitized female.

Intermediate stages between Figure 4G and Figure 4I were not found. It is very probable that such do not exist, in which case one must assume that the expo-

dite appears suddenly, following a particular molt, and is from the start long and well developed. The other alternative, that it requires more than one instar to produce a fully developed exopodite, is unlikely since Brachyuran crabs infested with sacculinids do not molt, as a rule, after the parasite has become external.

A final observation with respect to parasitized males needs to be mentioned, namely, that the concavity of the sternal plastron of the thorax is appreciably diminished, and the tubercles on the fifth sternal segment, which form a locking device to hold the abdomen flatly against the underside of the body, are greatly reduced if not entirely absent. These are both changes towards the female condition. The male gonopore is also reduced in size.

Modification of the Female Host

Female Callinectes parasitized by Loxothylacus show less extensive changes than the males. The most striking modification is that the abdomen becomes proportionally as broad or even broader than that of the normal adult female, although the size of the crab is such that normal specimens of that carapace width would still have the immature triangular abdomen (Fig. 2D–F). Of the 13 parasitized females, 8 were less than 60 mm. in carapace width (40, 42, 47, 48, 52, 52, 53 and 58 mm. respectively) and all were under 85 mm., yet each possessed a broad abdomen. The change from the triangular-shaped abdomen to a broad and rounded form does not take place in normal *Callinectes sapidus* until a carapace width of about 95–100 mm. has been reached (Newcombe, Sandoz and Rogers-Talbert, 1949). Females of that size are ready for the final molt which will reveal the form of the mature crab. Thereafter, females apparently do not molt (Churchill, 1919; Gray and Newcombe, 1939). It appears highly probable that in parasitized specimens the change in type of abdomen is likewise achieved at a single molt, but at a much earlier instar. The change is perhaps effected soon after the parasite has become established.

This precocious alteration in the shape of the abdomen of small parasitized females is a phenomenon which cannot be disregarded in attempts to explain the mechanism of parasitic castration. It parallels the cases of *Inachus mauretanicus* reported by Smith (1910) and of *Pachygrapsus marmoratus* reported by Pérez (1933), where the immature sacculinized females likewise assume prematurely the adult type of abdomen.

The pleopods of infested females are also altered to a greater or lesser degree. This involves a reduction in size of the internal ramus together with the basipodite, as shown in Figure 4A–D, and an apparent curvature of this arm away from the outer ramus so that the appendage becomes arcuate, in place of the normal Λ-shape. The expodite, on the other hand, does not seem to be affected in any significant manner.

As in the parasitized males and normal mature females, the "locking device" on the sternum of infested females is reduced or absent.

Discussion

In the introduction to this paper it was stated that an understanding of the abnormal may shed light on the normal. A good illustration of this is the manner

whereby the male copulatory appendage becomes altered into a female-like biramous pleopod. The highly specialized uniramous male appendage has been difficult to homologize with the fundamental biramous plan. Cochran (1935) acknowledged this in the following statement: "The name 'flagellum' is chosen arbitrarily for the distal part of the [male] pleopod, as it does not show the character of a true flagellum. But neither is there sufficient evidence for considering it a highly modified endopodite or exopodite." Snodgrass (1936) used the term "flagellum" or telopodite for this portion of the pleopod and Cronin (1947) has employed the same terminology. That the so-called flagellum is actually a modified endopodite is clear from the transformation which the second male pleopod undergoes in parasitized crabs (Fig. 4E–I). Reconstruction of a biramous type is achieved by a completely new outgrowth, the exopodite, while the original ramus remains as the endopodite.

The parasitized crab can help us solve another morphological puzzle, namely, how to homologize the two pairs of dorsal muscles in the fused section of the normal male abdomen with those of the female. The changes in musculature which the infected male undergoes make it probable that it is the third pair which is lacking in the normal male, and therefore the designations for the muscles of the male, as given by Cochran (1935), could be made more precise.

When we analyze the entire complex of changes imposed upon the male crab by parasitism, we cannot avoid the conclusion that these modifications are all manifestations of one primary process, namely, assumption of an intersexual condition or partial feminization. The change of the male abdomen from a state of partial rigidity to one of pliability, the effects of parasitism on the concavity of the sternal plastron and on the tubercles of the fifth sternal segment, the change in structure of the second pleopod and the increase in number of hairs around the margins of the abdomen, all these are feminine characteristics.

But the female Callinectes infested with Loxothylacus shows no approach towards the opposite sex. The abdomen does not become masculine in form, rather it responds to parasitism by becoming still broader. The female pleopods, on the other hand, neither assume the adult form concomitant with the change in shape of the abdomen, nor approach the condition in the male. In the normal male, the outer ramus is absent but in parasitized females it is the inner ramus that is atrophied.

Of the various theories that have been proposed to explain the nature and effects of sacculinization, we believe that Goldschmidt's theory (1931) fits the case presented here better than any of the others. Goldschmidt postulated that the quantitative balance between male and female sex substances present as a result of genic action in each individual was disturbed by the parasite, resulting in the characteristic modification. This explanation is based on his "theory of intersexuality." An individual becomes purely male or female when the substance produced by the M-gene or the substance produced by the F-gene exceeds the opposite by a certain quantitative value. In case the inequality between the two substances is not great enough to surpass the threshold, varying degrees of intersexuality will result. Moreover, an individual could develop as a male or a female up to a certain point of time, then, if for some reason an imbalance in the production of M or F substances occurred, development would switch over to the opposite sex and an intersex would be produced.

Goldschmidt regarded the modifications produced by rhizocephalids on their crustacean hosts as cases of "parasitic intersexuality." This appears to hold good for the parasitized males that exhibit feminine secondary sex characters, but not for such parasitized females as Inachus, Pachygrapsus, or Callinectes which are modified but not in the direction of intersexuality. In fact, Goldschmidt, although aware of the effects of parasitism on female Inachus, disregards the modified females entirely in his interpretation of the known phenomena of sacculinization.

Despite this omission, the theory of Goldschmidt need not be altered in its essentials to cover the case of the females. If, in a genetic male, the factor of parasitism enhances the reaction due to the F-gene or depresses that induced by the M-gene to the point where the normal relation became reversed and an intersex results, why should not the same factor operating on a female likewise increase the F-reaction or depress the M-reaction? The result in the latter case would be not intersexuality but a "strengthening" of femaleness. This reasoning provides a plausible explanation for sacculinized Inachus, Pachygrapsus and Callinectes insofar as the precocious development of certain adult characteristics in the immature female is concerned.

Exactly how the abnormal balance in the sex substances of the host is achieved by action of the parasite is a question difficult to answer. Reverberi (1944–45), who also made use of Goldschmidt's theory to explain the complex phenomena of parasitic castration, presents the suggestion that the parasite itself might elaborate a substance similar to F or activate in the host a superproduction of the F-substance; or conversely, it might take away the M-substance from the host or inhibit the production of the M-substance. Admittedly this is purely conjectural, but certainly within the realm of possibility.

Goldschmidt's theory, modified in the way in which we have just outlined it, appears to explain all the changes observed in parasitized Callinectes except for the strangely altered pleopods of the female. We shall now attempt to account for this exception.

First of all, the morphological nature of the altered female pleopods is such that one cannot describe them as retarded in development nor, strictly speaking, as approaching the normal male type. Neither can pressure against them by the external sac of the parasite explain their deformation, since various authors (Giard, 1887; Smith, 1906; Bulgurkov, 1938) have shown that sacculinized female crabs may display deformed swimmerets before the parasite becomes external.

Secondly, it is important to realize that the entire pleopod does not atrophy; the affected portion is the endopodite. The function of this ramus in normal crabs is an ovigerous one, while the external ramus is not concerned with the carrying of eggs. For this reason the endopodite may be regarded as a special type of accessory female sex structure whose normal development may be causally connected with the proper functioning of the ovary.

Le Roux (1931, 1931a) has shown that in female Gammarus the differentiation of certain secondary sex characters is determined by the functioning of the gonads, while that of others evolves independently of them. These results are in accord with those obtained by Haemmerli-Boveri (1926) on the females of the isopod Asellus, and by Callan (1940) on female prawns of the genus Leander. It is highly probable, therefore, that there exists in crustaceans an ovarian hormone which con-

trols the differentiation of certain structures associated with the care of developing young.

If then, in Callinectes, the differentiation of the inner rami of the pleopods is under the special influence of the gonads, atrophy of the sex glands as a result of parasitism would explain the failure of the endopodites to develop properly;[2] whereas the abdomen, whose morphogenesis may have no causal connection with this ovarian hormone, would evolve in a manner determined by the amount of F-substance present at a particular period of development.

Postulation of a feminizing stimulus elaborated by the parasite would not be contrary to the view maintained by us in an earlier paper, (Reinhard and von Brand, 1944) regarding toxic substances. Toxic agents could still be responsible for the atrophy of gonads and various physiological disturbances of the host. But the external morphological changes, which appear to operate chiefly in the same direction in both sexes, could well be determined in the manner just outlined.

Summary and Conclusions

Loxothylacus texanus Boschma, a rhizocephalid of the family Sacculinidae, infests the blue crab, *Callinectes sapidus* Rathbun, in the region of the Gulf of Mexico and causes alternation of the secondary sex characters of the host. The parasite attacks the smaller crabs that have not yet attained sexual maturity.

In accordance with the high degree of sexual dimorphism of the host species, the effects on the male are most striking since they tend to make it resemble the female. The narrow abdomen becomes broad and marginal hairs are increased. The third, fourth and fifth abdominal segments, previously fused, become freely movable; while the dorsal abdominal musculature comes to correspond with the altered segmentation. The second male pleopod shows varying degrees of modification resulting in two instances in a biramous type of appendage. The concavity of the sternal plastron, the tubercles on the fifth segment, and the gonopore are reduced.

In the immature female host, the triangular abdomen also assumes the broadness of the normal adult female, the sternal tubercles atrophy and the pleopods are altered to an arcuate form through curvature and shortening of the endopodite. No approach towards the opposite sex takes place in parasitized females.

The external modifications in parasitized male Callinectes clearly fall into the category of "parasitic intersexuality" and may be interpreted as resulting from a disturbance of the balance between male and female sex-formative substances brought about by the action of the parasite. The same type of parasitic activity, operating on a female host, should likewise increase the relative amount of female substance. Therefore immature parasitized Callinectes, instead of becoming intersexual, are hyperfeminized and develop precociously the adult female type of abdomen. The female pleopods, however, may constitute a special type of secondary sex character independent of the sex substances but dependent upon the normal maturation of the ovary. Suppression of ovarian growth by the parasite would then interfere with their proper development.

[2] Another instance of this is the case of *Pagurus longicarpus* recently reported by the writer (Reinhard, 1950, *Journ. Parasit.*, **36** (*2*) : 131–138) where the chief effect of parasitism by an entoniscid is the suppression of the normal development of the endopodites of the female crab.

These two-fold agencies, an upset in the normal relationships between the male and female sex-formative substances and the loss of ovarian control over the differentiation of the female pleopods, together give a plausible explanation of the external effects of sacculinization, particularly when applied to the case of Callinectes parasitized by Loxothylacus.

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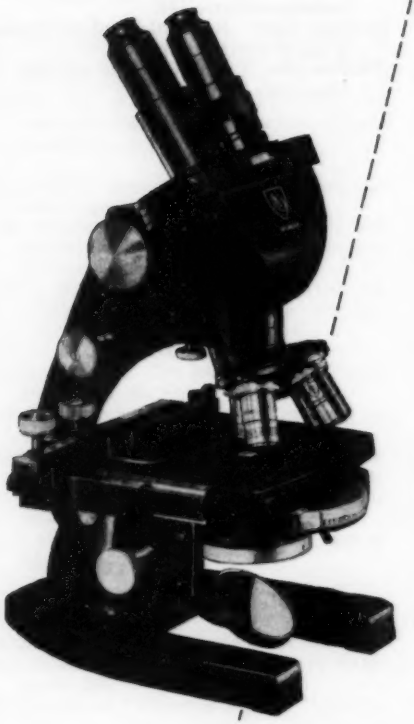

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